INVESTIGATION INTO ALTERNATIVES TO FETAL CALF SERUM IN ANIMAL CELL CULTURE

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Joanne Keegan Date: 27/6/94

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ABBREVIATIONS

AA	Amino acid
ACE	Acetone
ACN	Acetonitrile
Acyl-CoA	Acyl Coenzyme A
AMP	Adenosine monophosphate
AP	Acid phosphatase
apoTf	apo-transferrin
ATP	Adenosine tri-phosphate
BME	Basal Medium Eagle
BSA	Bovine serum albumin
BSA faf	Bovine serum albumin fatty acid free
BSE	Bovine spongiform encephalopathy
CIV	Collagen type IV
cAMP	cyclic adenosine mono-phosphate
CC	Cell count
CHOK1	Chinese Hamster Ovary K-1
$CHCl_3$	Chloroform
CN	Cell number
COOH	Carboxyl
CO_2	Carbon dioxide
CPSR	Controlled process serum replacements
CT	Cholera toxin
CV	Coefficient of variation
DE	Dye elution
DEE	Di-ethyl-ether
Dex	Dexamethazone
DHS	Donor horse serum
dhfr-MTX	di-hydrofolate reductase - Metothrexate
DNA	Deoxyrebonucleic acid
DME	Dulbecco's modified Eagle's medium
DMSO	Di-methyl sulfoximide
EA	Ethanolamine
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPO	Erythropoietin
eRDF	enhanced RDF (RPMI DME Ham's F12) medium
ßES	ß-Estradiol
ESA	Equine serum albumin
ESA faf	Equine serum albumin fatty acid free
ESG	Ewing's sarcoma growth factor
EtOH	ethanol
FAC	Ferric ammonium citrate
FAS	Ferric ammonium sulfate
Fbn	fibronectin
FBS	Fetal bovine serum
FC	Ferric citrate
FCS	Fetal calf serum
FePIH	Ferric pyridoxal isomcotinoyl hydrazone
FeSIH	Ferric salicylaldehyde isomcotinoyl hydrazone
FGF	Fibroblast growth factor

FN	Ferric nitrate
Fu	Fetuin
GH	Growth hormone
GL	Glycogen
GMP	guanosine mono-phosphate
GTP	guanosine tri-phosphate
HAT	Hypoxanthine - Aminopterin - thyroxine
HBGF	Heparin binding growth factor
HbsAg	Hepatitis b surface antigen
HC	Hydrocortisone
HDL	High density lipoprotein
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	High performance liquid chromatography
HS	Heparin sepharose
HSA	Human serum albumin
HSA faf	Human serum albumin fatty acid free
IA	Image analysis
ICC	Iron choline citrate
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
INF	Interferon
Ins	Insulin
Ins-R	Insulin receptor
IMDM	Iscove's modified DME
IPSF	Immunoglobulin production stimulating factor
IRE	Iron responsive element
IRE-BP	Iron responsive element - binding protein
IRS	Insulin receptor substrate
kDa	kilodalton
LA	Linoleic acid
LC	Liquid chromatography
LDL	Low density lipoprotein
L-Glut	l-glutamine
LH	Luteinizing hormone
LM	laminin
LP	lipoprotein
LPA	lysophosphatidic acid
LPSR	low protein serum replacements
mA	milli-Amps
mAbs	monoclonal antibodies
MAP-kinase	Microtubule associated protein kinase
M-CSF	Macrophage colony stimulating factor
MDCK	Madm-Darby canine kidney
ß-ME	ß-mercaptoethanol
MeOH	Methanol
MEM	minimal essential medium
mRNA	messanger RNA
M6P	Mannose-6-phosphate
NADH	Nicotinamide adenine dinucleotide
Na-K	Sodium-potassium
Napi	Sodium phosphate
NCTCC	National Cell and Tissue Culture Centre

NEAA	Non-essentıal amıno acıds
NRK	Normal rat kıdney
NSCLC	Non-small cell lung cancer
O	Oleıc acıd
OD	Optıcal densıty
P	Pyruvate
PA	Phosphatıdıc acıd
Pal	Palmıtıc acıd
PBS	Phosphate buffered salıne
PEA	Phosphoethanolamıne
PDGF	Platelet-derıved growth factor
PGE	Prostaglandın
PNP	Para-nıtro-phenol
Prl	Prolactın
Pu	Putrescıne
RGD	Arg-Gly-Asp, based on alphabetıcal code for amıno acıds
RME	Receptor medıated endocytosıs
RNA	Rıbonucleıc acıd
rp-HPLC	reverse phase HPLC
SCC-9	Squamous cell carcınoma-9
SDS-PAGE	Sodıum dodecyl sulfate - polyacrylamıde gel electrophoresıs
Sel	selenıum
SFM	Serum-free medıum/medıa
SNP	Sodıum mtroprussıde
SSM	Serum-supplemented medıum
T	Trııodothreomne
TE	Trace elements
TEMED	N,N,N',N',-Tetramethyl-ethylenedıamıne
Tf	Transferrın
TFA	Trıfluoroacetıc acıd
TGF	Transformıng growth factor
TI	Trypsın ınhıbıtor
TNF	Tumour necrosıs factor
TSH	Thyroıd stımulatıng hormone
tRNA	transfer RNA
TV	Trypsm versene
UP	Ultrapure
UV	Ultravıolet
Ve	Elutıon volume
Vo	Voıd volume
w/v	weıght/volume

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

Mammalian cell culture has wide and expanding applications in both research and biotechnological/pharmaceutical industries The development and routine use of serum-free media (SFM) is a high priority for cell culture from both an industrial and scientific point of view as evidenced by the literature and the ever increasing supply of commercial preparations designed to bulk up growth in serum-free systems Production of vaccines, therapeutics and monoclonal antibodies for diagnostic and therapeutic purposes are just some of the many applications in which mammalian cell culture can dominate

For cell culture products to be of diagnostic and therapeutic use, the quality of these biomaterials need to meet the highest standards In addition to precise process control during isolation of the product, the milieu in which the cells are active is of prime importance

The complexity and undefined nature of serum has been recognised for some time Several approaches have been taken to deal with this, including reduction or total elimination of serum from the medium With the development of SFM, problems have been encountered Cell types exhibit different nutritional and supplemental requirements With the amount of variables in ever increasing combinations and numbers that can be used, the task of finding a SFM for a particular cell line is daunting

In this literature survey, the problems associated with the use of serum and the development of more defined culture conditions are discussed An overview of the factors generally used in the replacement of serum are presented The possible advantages of using serum-free medium on an industrial scale are investigated An outline of commercially available serum-free medium is given
A detailed review of three of the most commonly used factors in serum-free medium, insulin, transferrin and bovine serum albumin (BSA) is presented with a view to replacing these components with non-animal-derived products in order to develop more defined media

1.1. BACKGROUND

In vitro, the external medium must be able to provide all the requirements that cells would normally obtain *in vivo* for growth. In the early stages of animal cell culture, it was discovered that a small amount of serum in the basal medium would support the growth and proliferation of cells. Other body fluids such as amniotic fluid (Barnes and Sato, 1980a), milk and chicken egg yolk (Fujii and Gospodarowicz, 1983) and bovine colostrum (Pakkanen *et al.*,1992) were tried but serum proved to be the most efficient at supporting growth. The most commonly used serum was/is fetal calf serum (FCS) because of its effectiveness. It typically constitutes 5-10% vol/vol of the growth medium for cells.

1.1.1 Problems with FCS

There are disadvantages associated with the use of serum, especially FCS. It is not adequately chemically defined. For most cells, serum is not the physiological fluid in which the cells are maintained as it contains many factors released *in vivo* during wound healing and blood clotting processes. Indeed, cells grown regularly in serum-supplemented medium may change by adaption or selection from the primary cells from which they were originally derived.

Studies of the mechanisms and actions of hormones and growth factors are hampered by the presence of serum (use of charcoal-treated or heat-inactivated serum has been used to study hormonal and growth factor actions respectively (Hayashi and Sato, 1979; Price and Gregory, 1982)). The interaction of hormones, growth and attachment factors is hard to detail due to the ill-defined nutritional environment of cells.

Serum is a potential source of toxins and growth inhibitors (chalones, selective inhibitors and toxins, for example, polyamine oxidase which catalyses the formation of toxic polyaminoaldehydes from polyamines is present in FCS (Allen *et al.*, 1979)). Serum is a potential source of bacterial, viral and mycoplasma contamination *e.g.* bovine spongiform encephalopathy (BSE) and mycoplasma (Phillpotts, 1989; Hodgson, 1990; Sasaki *et al.*, 1984). In addition to mycoplasma using up components of the medium required by cells, mycoplasma products such as arginine deaminase often have detrimental effects on cell growth (Sasaki *et al.*, 1984). Transforming Growth Factor - ß (TGF-ß), which is present in serum, is growth inhibitory to many cells of epithelial and neuroectodermal origin (Miyazaki and Horio, 1989). The presence of TGF-ß may reduce the growth stimulatory ability of serum. Berthier *et al.*

(1993) found human megakaryocyte progenitor cells to grow better in SFM than in plasma-supplemented medium There was an increase in growth in the plasma-supplemented media on the incorporation of TGF-ß neutralizing antibodies Other factors present in serum including glucocorticoids and serum lipoproteins (Ito *et al* , 1982) have also been found to exert inhibitory action over some cell lines In addition, serum components can cause undesirable effects such as cellular differentiation as seen with murine megakaryocyte progenitor cells (Tsukada *et al* , 1992)

Serum can be costly In a large-scale operation, FCS can account for up to 84% of the media costs (Griffiths, 1986) However the cost of using SFM will depend on the ability of the cells to grow in defined conditions Some cells require the introduction of growth factor combinations in addition to other usual components ever present in the medium, thus resulting in more expensive alternatives to serum, while other cells which produce their own growth factors will grow in a relatively inexpensive SFM Hybridomas and myelomas have been found in general, to be less fastidious and will grow in SFM with minimal additions and little dependence on growth factors

Serum suffers from batch-to-batch variations in biological activity, protein concentrations *etc* , and this can cause significant problems on an industrial scale In addition to inter-batch variations, fluctuations in commercial availability (demand often exceeds supply) can drastically affect the production costs Serum can also act as a source of potentially contaminating antigens in monoclonal antibody production The use of donor serum, for example Donor Horse Serum (DHS) can partially alleviate some of these problems The fact that the animal is not killed means that a well controlled herd can be used to supply a large amount of serum over a period of time and as a result there are no limitations to availability The use of a 'disease free' herd and clean handling of sera can reduce potential contaminants

1.1.2 Development of serum-free/serum-reduced media

There are many detailed reviews which outline the progress made in the development of SFM (Barnes and Sato, 1980b, Mather, 1984, Hewlett *et al* , 1989), while other reviews focus on growth of specific cell types in SFM (Miyazaki *et al* , 1984, Bjare, 1992, Sandstrom *et al* , 1994)

In the formulation of a SFM, there are two main points to consider, the basal nutrient medium and the supplements (Dulbecco, 1970) The composition of both may vary from cell type to cell type and also on the desired result Basal nutrient media are multifactored providing most of the nutrients required by the cell These factors include amino acids (both essential and non-essential), vitamins (especially B group), nucleic acids, lipids (essential fatty acids, glycerides *etc*), inorganic salts (as buffering agents, co-enzymes and co-factors) and an energy source (usually glucose or fructose) Addition of serum then provides factors not included in the basal media Factors include hormones / growth factors, attachment factors, transport proteins and detoxifying agents, purines and primidines, nutrients/energy source, protease inhibitors, trace elements of organic and inorganic compounds Basal media are not always optimized for a particular cell line, so addition of vitamins, amino acids or trace elements may be required for optimizing growth Developing the basal medium for a particular cell line can reduce the number of additional serum-free components However, due to the extent of factors in serum, it has not been possible to identify them all and develop a universal SFM which will provide the support required for the majority of cell lines In general, the more defined a SFM becomes, the more specific it becomes for a given cell line

A number of different approaches to replace FCS have been undertaken The amount of serum in the medium can be reduced or replaced by a less expensive undefined components Eagle (1955) developed a basal medium which would support the minimum growth of cells (Eagles MEM) This medium consisted of 13 ammo acids, 8 vitamins, 6 ionic species and glucose Supplementation with dialysed serum allowed the growth of L and HeLa cells Guilbert and Iscove (1976) were able to reduce the serum level from 15% to 1% with the incorporation of transferrin, sodium selenite, BSA and lecithin to grow erythroid cells derived from BDF mice Peehl and Ham (1979) used small amounts of dialysed fetal bovine serum with hydrocortisone to stimulate growth of human epidermal keratmocytes Shipley and Ham (1981) grew Swiss mouse 3T3 cells in medium with a serum protein concentration as low as 125μg/ml
Use of alternative sera which are cheaper and suffer less from batch-to-batch variation (including bovine, new born calf serum, human (Emerman *et al* , 1987), equine (McKeehan *et al* 1982), porcine and lamb serum) have also been investigated

Two broad categories of SFM can be distinguished on the basis of cell type and requirement for substratum adherence Anchorage-independent cells (hybridomas and myelomas) are in general, less fastidious than many of the anchorage-dependent cell types It has been easier,

not only to develop SFM but to design low protein (Blasey and Winzer, 1989) or protein-free media (Kovar, 1989, Darfler, 1990, Cleveland *et al* , 1983) which support growth and monoclonal antibody production In addition to reducing costs and having more definition, the use of low protein/protein-free media also means less problems in downstream processing Not only are most of these SFM totally defined, they are also relatively cheap, usually comprising low molecular weight components and defined polymers with no added proteins and so are ideally suited to industrial use However, these SFM are limited to a few established cell lines

Anchorage-dependent cell lines often require growth factors and attachment factors as well as other nutrients and trace elements In general, transformed cell lines have simpler requirements than untransformed cells Some systems have been developed to allow long-term growth in SFM by either transforming cells with carcinogens *e g* benz[a]pyrene (Stampfer and Bartley, 1985) or transfecting cells with oncogenes (Reddel *et al* , 1988)

The tissue from which the cell line has been derived may also affect the ability of the cell to grow in SFM While primary cultures from colon, mammary and lung require growth factors and attachment factors, many hepatoma primary cell lines can be cultured without such additives due to the large number of factors produced and secreted by both normal and neoplastic hepatocytes (Yano *et al* , 1986, Dufresne *et al* , 1993) This reflects the dynamic *in vivo* situation of hepatic cells

Table 1 1 shows some of the many SFM developed Insulin, transferrin and selenium appear most often A wide range of growth factors (including EGF, PDGF, IGF, FGFs and TGFs), hormones (dexamethazone, hydrocortisone, ß-estradiol and prostaglandins) and attachment factors (fibronectin, collagen and laminin) are listed

Table 1.1

REFERENCE	CELL LINE	BASAL MEDIUM	Ins µg/ml	Tf µg/ml	BSA mg/ml	SEL (nM)
Iscove and Melchers (1978)	Fo, NS-1, Sp2/0	IMDM	--	1	0 5	100
Chang *et al* (1980)	Ag8	MEM	5	5	--	--
Murakami *et al* (1982)	MPC 11, NS-1, SP2/0	ATCC	5	35	--	2 5
Chiang *et al* (1985)	NIH 3T3	ATCC	10	25	--	--
Hosoi *et al* (1991)	Bovine Granulosa cells	ATCC	5	--	25	--
Yang *et al* (1982)	primary normal mammary epithelial cells	ATCC	10	10	1 0	--
Kawamoto *et al* (1983a)	NS-1	RPMI F12 DME(2 1 1)	10	10	0 5	1
De Boer *et al* (1989)	Splenocytes	DMEM	5	36	2 5	--
Morrison and De Vellis, (1981)	Astrocytes	ATCC	50	--	--	--
Monette *et al* (1990)	Erythroid stem cells	MEMα	10	--	10	--
Johnson *et al* (1992)	Normal Human Keratinocytes	DMEM	5	5	0 02	53
Hutchings and Sato (1978)	HeLa	Ham's F12	5	5	--	15
Darlington *et al* (1987)	Human Hepatoma	MEM/MAB (3 1)	10	10	--	30
Branchaud *et al* (1990)	Human Placental Trophoblasts	RPMI	15	10	0 7	--
Anver *et al* (1982)	Mouse Metanephric organs	ATCC	5	5	--	--
Briand *et al* (1987)	Human primary mammary epithelial	ATCC	0 25	10	--	2 6ng/ml
Gazdar and Oie (1986)	Human primary NSCLC	RPMI-1640 or ATCC	20	10	2 0	25nM
Allen *et al* (1985)	Satellite Cells	MCDB-104	10^{-6}M	5	1	30
Taub *et al* (1979)	Kidney cells	ATCC	5	5	--	10

Abbreviations Ins = Insulin, Tf = Transferrin, Sel = Selenium, NEAA = Non Essential Amino Acids, GF = Growth Factor, ßME = ßMercaptoethanol, EA = Ethanolamine, LA = Linoleic acid, O = Oleic acid, P = Pyruvate, Gl = Glucagon, Pu = Putrescine, HC = Hydrocortisone, Dex = Dexamethazone, Pal = Palmitic acid, PEA = Phosphoethanolamine, T = Triiodothreonine, Fu = Fetuin, EPO = Erythropoietin, Il = Interleukin, ATCC = 50 50 vol/vol DME Ham's F12, Fbn = Fibronectin, HBGF-2 = Heparin binding growth factor-2, Lp = Lipoprotein, EGF = Epidermal growth factor, CT = Cholera toxin, PDGF = Platelet-derived growth factor, FGF = Fibroblast growth factor, C IV = Collagen type IV, Prl = Prolactin

Table 1.1 continued

REFERENCE·	GROWTH FACTORS	HORMONES	TRACE ELEMENTS	OTHERS
Iscove and Melchers(1978)	--	--	--	50μM ßME
Chang *et al* (1980)	--	--	--	NEAA
Murakami *et al* (1982)	Pu	--	20μM EA	0 5mM P 40ng/ml LA
Chiang *et al* (1985)	10ng/ml EGF	--	--	5μg/ml Fbn 25μg/ml HDL
Hosoi *et al* (1991)	500ng/ml Aprotinin 10ng/ml HBGF-2	--	--	25μg/ml Lp
Yang *et al* (1982)	10ng/ml EGF	1μg/ml Cortisol	--	10ng/ml CT Collagen Gel Matrix
Kawamoto *et al* (1983a)	--	--	10μM EA	1mM P
De Boer *et al* (1989)	--	--	20μM EA	1μg/ml LA 1μg/ml O 1μg/ml Pal
Morrison and De Vellis (1981)	100nM Pu 500ng/ml PDGF 100ng/ml FGF	50nM HC	--	--
Monette *et al* (1990)	2U/ml EPO 50U/ml IL-3	--	--	0 1mM ßME NEAA
Johnson *et al* (1992)	10ng/ml	0 4μg/ml HC 20pM T	10mM $SnCl_2$	0 1mM EA 0 1mM PEA
Hutchings *et al* (1978)	30ng/ml EGF	30mM HC	$N_{i_2}SO_4$, $MnCl_2$, $NH_4Mo_2O_7$,$SnCl_2$, He_2SO_4, $CdSO_4$ Na_2SiO_3,$NaHVO_4$,	--
Darlington *et al* (1987)	--	1μg/ml Gl, GH 1μM T, Dex	--	1μg/ml LA
Branchaud *et al* (1990)	--	--	--	1-2μg/ml LDL
Avner *et al* (1982)	25ng/ml PGE	3 2pg/mlT 5μg/ml HC	--	--
Briand *et al* (1987)	100ng/ml EGF	1μM HC 5μg/ml Prl 0 1nM ßEs	--	2mM 1-Glut C IV
Gazdar and Oie (1986)	1ng/ml EGF	50nM HC 100pM T	0 5M P 10μM EA, PE	2mM 1-Glut C IV
Allen *et al* (1985)	0 5mg/ml Fu 100ng/mlFGF	100nM Dex	--	--
Taub *et al* (1979)	25ng/ml PGE	50pM T 50nM HC	--	--

1.1.3 Advantages to growing cells in SFM

There are many advantages to growing cells in serum-free conditions As a defined system, the use of SFM can be manipulated to obtain growth of a particular cell line from a mixture of cells *i e* selective growth, which takes advantage of different rates of cellular attachment to a substratum or different growth rates (Suzuki *et al* , 1989, Birkenfeld *et al* , 1988, Kuneko and Goshina, 1982) This is typically seen in primary cultures where undesirable fibroblast contamination can prevent the isolation of epithelial cells (Bottenstein and Sato, 1978) As epithelial cells attach more slowly than fibroblastic cells to a substratum, epithelial cells can be easily removed by slight agitation or very short exposure to trypsin Selective growth has been used with a variety of cell types including primary culture of embryonic chick dorsal root ganglia (Barnes and Sato, 1980b), ovarian epithelial cells (Orly *et al* , 1980) and human non-small cell lung cancer cells (Brower *et al* , 1986) SFM have also been used to permit selective growth of tumours from fresh clinical specimens (Van der Basch, 1984, and Carney *et al* , 1984)

Yabe *et al* (1986), developed a SFM for the establishment and selection of mouse hybridomas without the presence of HAT (hypoxanthine, aminopterin and thymidine) called NYSF-404 The rate of hybridoma formation was found to be twice as good as that observed using traditional cloning techniques requiring serum and HAT-supplemented medium

Other means of selection based on the selective depletion of specific factors from the medium have also been developed Hepatocytes can be isolated from a liver culture using the conversion of L-ornithine to L-argmine as a basis of selection By using arginine-free ornithine containing medium, only hepatocytes (which contain transcarboxyamylase) will grow (Salas-Prato, 1982) Use of D-valine instead of L-valine in SFM preferentially allowed the growth of human and rodent epithelial cells (Gilbert and Migeon, 1975) The fibroblasts, which lack the essential ammo acid oxidase, cannot convert D-valine to L-valine and thus cannot grow However, this may not be true for all fibroblasts as it was recently reported that anterior pituitary adenoma fibroblast cells were not inhibited in serum-free medium containing D-valine (Masson *et al* , 1993)

Beattie *et al* (1990) used a specific cytotoxin to cells expressing the basic fibroblast growth factor (ß-FGF) receptor by conjugating the growth factor to Saporin-6, a ribosome-inactivating protein for 96 hours. This allowed pancreatic islet cell growth in the absence of fibroblasts

Chemically defined media may be used to control the differentiation of cells (Rahenchilla *et al* , 1989, Reyne *et al* , 1989, Najar *et al* , 1990) For example, calcium ions control the differentiation of normal human keratinocytes in SFM (Pillai *et al* , 1990)

Serum was routinely used in combination with di-methyl-sulphoxide (DMSO) to protect cells during cryopreservation Methylcellulose, casein and various SFM have been used to replace serum to allow 'defined' cryopreservation of cells grown in SFM (Yoshida and Takeuchi, 1991, Li *et al* , 1993, Johnson *et al* , 1992) This means that cells need not be contaminated with serum components during cryopreservation

1.1.4 Problems with Serum-free media

The development of SFM for anchorage-dependent cells has had varied success Some cell lines like HeLa (Hutchings and Sato, 1978) and MDCK (Taub *et al* , 1979) have been found to grow as well in SFM as in serum-supplemented medium even over extended periods (many subcultures) Continuous growth in SFM has been reported for other cell lines where growth is only slightly less than that obtained in serum-supplemented medium including HT-29 colon carcinoma and TWI melanoma cells (Zirvi *et al* , 1986), Oat cell carcinoma cells (Simms *et al* , 1980) and non-malignant human squamous cells (Rikimaru *et al* , 1990) In some cases however, although long-term subculture has been reported, the use of serum or Pedersons Fetuin to inactivate trypsin while subculturing, means that there may be some residual serum being carried through with each successive passage (Oda and Watson, 1990, Malan-Shibley and Iype, 1983)

However, the success with anchorage-dependent cells is not as extensive Many of the anchorage-dependent cells are more fastidious growers and a simple combination of factors cannot totally replace serum As a result, growth is often slower in SFM (Ahearn *et al* , 1992) In some cases 'SFM' have been developed where the cells must first be plated in medium containing serum and allowed to attach The supernatant is then removed and replaced by a SFM which supports growth (Jozan *et al* , 1992)

Use of ill-defined components to supplement media includes Pedersens Fetuin (Elliot and Auersperg, 1993), bovine brain extract (Maciag, 1981), crude membrane extracts (Saad *et al* ,

1993) and bovine pituitary extract (Bertolero *et al* ,1984, Wille *et al* , 1984, Kirk *et al* , 1985, Gilchrest *et al* , 1982 and Peehl and Stamey, 1986) Use of peptones as serum substitutes reduce costs and the possibility of contamination because they are autoclavable (Rutzky, 1981)

Adaption of cells to growing in a SFM often occurs This may involve the initiation of autocrine systems, synthesis of components of the extracellular matrix or using initial substrates of biosynthetic pathways instead of the intermediates normally provided by serum As a result of these activities, a slower initial growth of cells in SFM will be seen followed by sustained growth The use of high cell densities is often required so as not to dilute down autocrine factors beyond a critical level

Kaighn *et al* (1988) looked at changes in the karyotype of two mouse keratinocytes, MK1 and MKDC4, to see what effect growth in SFM would have MK1 and MKDC4 cells could undergo 400 and 200 doublings respectively but significant karyotypic changes occurred at passages 4 and 7 The cell chromosome number increased from diploid to near tetraploid with less dependence on bovine pituitary extract after several doublings This corresponded to the time taken for the cells to adapt to growing in SFM Guhe and Follmann (1994) when investigating the characteristics of porcine urinary bladder epithelial cells found that for up to 4 weeks that cells exhibited similar marker enzymes and that after the first 5 weeks, there was an increase in polyploid and polynucleated cells Jefferson *et al* (1985) noticed considerable changes in protein synthetic patterns in MDCK cells between passages 1 and 10 in SFM

Retention of normal cellular functioning is important especially when a product is desired as in the case of hybridoma production of monoclonal antibodies MDCK and Opossum kidney retained their ability to form hemicysts, while Hela-S_3 continued to produce α-subunit of glycoprotein hormones in a SFM but after 4 days production was only 50-80% of that seen in serum-supplemented medium (Taub *et al* , 1979, Leiderman *et al* , 1989, Morrow *et al* , 1981)

1.2 MEDIA SUPPLEMENTS

Cellular requirements will differ depending on the following the embryonic origin of the cells (ecto, meso or endodermal), species specific requirements, and whether the cells are normal or transformed Therefore the media supplements required will vary from cell type to cell type In addition, many cells grown in SFM are much more density-dependent than cells grown in serum-supplemented medium As early as 1962, Eagle and Piez found that cultured mammalian cells synthesised serine but if the cell density was too low, the amount of serine released to the medium was insufficient for the survival of cells

Media supplements can be categorised as follows transporters, growth promoters (hormones and growth factors), attachment factors, vitamins, trace elements and lipids

Transporter molecules include transferrin and albumin which transport iron and most lipids respectively These will be discussed in more detail in section 1 6 and 1 7

1.2.1 Growth Promoters

Dulbecco (1970), found that many cells exhibited a low level dependence on the serum concentration for the initiation of DNA synthesis but were strongly dependent on the serum level to undergo mitosis Proliferation and differentiation of animal cells is regulated by the events leading to DNA synthesis *i e* the exit from the G1 phase and entry into the S phase This is controlled by external conditions affecting cells Hormones and growth factors which are present in serum are known to exert control over cell division Such factors must be incorporated into many SFM to allow replication of cells

Growth promoters include insulin, IGFs, EGF, PDGF, FGF, estradiol, dexamethazone and prostaglandins Insulin is used almost universally in SFM and its role is discussed later Most growth factors present in serum are in ng/ml concentrations Detailed information about growth factors can be found in Ham (1981), Habemcht (1990) and the 1994 R and D Systems catalogue (R and D Systems Europe)

1.2.1.1 Epidermal Growth Factor (EGF)

EGF is a 6kDa protein with a single peptide of 53 amino acid residues It is found in all body fluids under normal physiological conditions The almost universal importance is seen in its incorporation into most SFM EGF works by increasing the cell cycling fraction as observed in human diploid fibroblasts (increasing the move of cells from the G1 phase into the S phase) It can operate synergistically with insulin or IGF-I by reducing the lag time before DNA synthesis for the growth of Balb/c 3T3 cells (Pledger *et al* 1978, Brown and Holley, 1979)

1.2.1.2 Platelet-derived Growth factor (PDGF)

PDGF is a serum protein derived from platelets (α-granules of platelets) during the clotting process (Stiles, 1983) It is a heterodimer of 30kDa which may consist of α and/or ß chains depending on the species It is extremely cationic and hydrophobic and as such requires a carrier protein (usually BSA) for proper functioning It is a potent mitogen for connective tissue smooth muscle cells, fibroblasts and glial cells (Koher and Lipton, 1974) It stimulates the transition from G1 to S phase but does not increase the cell cycling fraction It also stimulates ammo acid uptake, the Na-K pump and increases the number of somatomedin surface receptors PDGF has been produced in an autocrine fashion by endothelial cells (Dicohleto and Bowen-Pope, 1983), osteosarcoma cells (Heldin *et al* 1980), and SV-40 transformed BHK cells (Dicher *et al* , 1981) When PDGF was incorporated into the medium, a lower level of EGF was required for growth of mouse CBH/10T1/2 cells (Carpenter and Cohen, 1979)

1.2.1.3 Fibroblast Growth Factors (FGF)

Acidic and basic FGFs are single chain polypeptides with molecular weights of 16 and 14 5kDa respectively They were originally isolated from bovine brain extract (Gospodarowicz *et al* , 1982) They are potent mitogens for endothelial, mesoderm and neuroectoderm-derived cells Both have been used to stimulate the growth of normal human keratinocytes and fibroblasts in SFM (Shipley *et al* , 1989) Heparin sulphate has been implicated in stabilizing the activity of α-FGF

1.2.1.4 Hormones

Hormones will have different effects depending on the cell system Steroid hormones like estrogen were shown to stimulate growth of human endometrial cells ß-estradiol stimulated growth by reducing the length of the G phase, much like insulin Estrogen in a SFM was shown to exert a mitogenic effect on MCF-7 cells but not on Ishikawa cells (Huff *et al* , 1986,

Dickson *et al* , 1986) Not only is varied response seen depending on the cell line, the basal medium may also dictate activity Holinka *et al* (1989) found that when minimal essential media (MEM) was used as the basal medium, no response to estradiol was seen, regardless of concentration, but when Earls basal medium (BME) was used, stimulation by estradiol occurred Nishizawa *et al* (1989) found that androgen and estrogen enhanced growth through unique receptors on transformed mouse leydig cells For human mammary breast cells, oestrogenic hormones were found to stimulate growth while androgens inhibited growth (Maclndoe and Eitre, 1980) The converse has been found for prostate cancerous cells Mice mammary tumour (Shiongi carcinoma 115) cells were markedly stimulated by androgen and estrogens (Noguchi *et al* , 1985) This indicates that hormonal interactions with cells may be species specific or site specific in their interactions

Glucocorticoids can have a variety of effects depending on the cell type and the presence of other factors They can modulate cell proliferation by altering the cells' responsiveness to other hormones or growth factors (Baker *et al* , 1978) For example, dexamethazone acted synergistically with IGF-I to stimulate the growth of smooth muscle cells (Conover *et al* , 1983) and cardiac myocytes (Suzuki *et al* , 1989) but down-regulated the insulin receptor substrate-I in 3T3-L1 adipocytes (Turnbow *et al* , 1994) Glucocorticoids are also involved in modulating the production of metabolites in a SFM (Coezy *et al* ,1984, Beuhnick and Cassio, 1983 and Togami *et al* ,1988) and growth promoting factors such as IGF binding proteins (Okazakai *et al* , 1994) The use of serum-free medium increased the sensitivity of CEM-C7 cells to dexamethazone (Chilton *et al* , 1990)

For other cell lines, autocrine production becomes turned on, enabling the cells to survive in defined medium with little or no additional growth promoters required (Matsuda *et al* , 1989, Soma and Grotendorst, 1989) Human skin fibroblasts were observed to produce an IGF-like peptide (Clemmons, 1984) while Reuber H-35 rat hepatoma cells were found to secrete transferrin in SFM (Shapiro and Wagner, 1989) The extent of autocrine production varies Human non-functional pituitary adenoma cells were found to produce EGF, TGF-α, IGF and ß-FGF (Renner *et al* , 1993) and human non small cell lung carcinoma cells were found to produce ß-FGF, IGF-I and II, EGF, TGF-ß2, TNF-α, TGF-ß1 and PDGF (Occleston and Walker, 1993) ß-FGF is produced by renal carcinoma cells (Mydlo *et al* , 1993) and human osteosarcoma cells (Nishikawa *et al* , 1993)

Transformed cells like Swiss 3T3 and Balb/c 3T3 have a lower requirement for serum (Clark, 1970, Dulbecco, 1970) The loss or reduction in requirement of exogenous growth factors may be due to an increase the number of high affinity cell surface receptors (Cherington and Purdee, 1980), production of transforming growth factors or alterations in the ability of cell surface receptors to initiate signal transduction *etc*

1.2.2 Attachment factors

The attachment of cells to their surrounding is important in determining cell shape and in maintaining proper cell function and tissue integrity Serum provides required attachment factors such as fibronectin and vitronectin which allow the cells to attach quickly Most cells are capable of producing attachment factors but attachment is in general, greatly stimulated by the presence of exogenous attachment factors So, the absence of serum from media not only affects growth and differentiation but has consequences for cell attachment The development of SFM and the initial widespread use of attachment factors has allowed a greater understanding of the chemistry and mechanisms of cellular adhesion and attachment, embryogenesis, morphogenesis, homeostatic processes, organ stability and tumour metastasis

Due to the fact that cells can synthesise attachment factors and all or most of the requirements for the extracellular matrix (ECM), many SFM no longer incorporate exogenous attachment factors but depend on the capabilities of the cells (Taub *et al* , 1979, Morita *et al* , 1993, Golombick *et al* , 1990, Hahm *et al* , 1990) However, addition of ECM components or attachment factors have been found to be necessary for the long-term growth of some established and primary cell lines (Gospodarowicz *et al* , 1981, Bridges *et al* , 1993, Baeza-Squiban *et al* , 1994) as these cells could not produce sufficient components to form their own ECM

When exogenous attachment factors are provided or when the cells synthesise attachment factors, an ECM is deposited The ECM is a complex network of secreted proteins and carbohydrates that fill the spaces between cells *in vivo* and between the cell and substratum *in vitro* which consists of a combination of collagens, proteoglycans and various glycoproteins such as fibronectin and laminin The ECM can act as positive or negative regulators of cell growth and/or differentiation, by interacting with hormones and growth factors (Tokiwa *et al* , 1988, Sarubbi *et al* , 1990, Lin and Bissell, 1993, Nagano *et al* , 1993, Nugent and Newman, 1989)

Two forms of adhesion were identified The first involves electrostatic interactions between the substratum (surface) and the cell, a process known as passive adhesion The second involves binding of the cell to a substratum *via* a protein coat (typically provided by serum) This is referred to as active adhesion

In passive adhesion, negatively charged cytoplasmic micro-extensions from the cell bind to a positively charged substratum The positively charged substratum can be achieved by chemical etching or physical treatment, *e g* , bombarding with electrons (Grinnell, 1978, Ramierez *et al* , 1984) or use of basic polymers such as polylysine, protamine or polyarginine (McKeehan and Ham, 1976) This provides a positive charge at physiological pH to which the cells can attach The use of negative charges has also been reported but the basis of adhesion is unknown since cells have a net negative charge at physiological pH (Maroudas, 1975) Serum factors or proteins inhibit passive adhesion by binding to the surface first and preventing electrostatic interactions between the cell and substratum

Active adhesion requires specific co-factors (usually supplied by serum), cytoplasmic components and particular cell surface receptors It is a slower process than passive adhesion, is energy dependent and requires cations (Mg^{2+}, Ca^{2+} and sometimes Mn^{2+}) It involves the laying down of the ECM, a general reorganisation of microtubules and microfilaments that comprise the cytoskeleton, and binding, *via* specific receptors to the ECM Serum components known to be involved include collagen, fibronectin and vitronectin These factors are all glycoproteins and most have been found to contain a specific sequence for recognition on the cell surface The site contains the short amino acid sequence Arg-Gly-Asp which is called the RGD (based on the alphabetical code for amino acids) site and is necessary for active binding RGD sequences have been found on vitronectin and lamimn (Cheresh *et al* , 1989), thrombospondin, fibrinogen, collagen and osteopoitin (Ruoslahti 1987, 1988), and on platelet-derived glycoprotein $GpII_B/III_a$ (Pytela *et al* , 1986) These all belong to a super family of factors, the Integrin family (Hynes, 1987) Differences in specificity of the factors is thought to be caused by neighbouring ammo acid sequences

1.2.2.1 Collagen

Of these factors, collagen is the most abundant protein in the basement membrane of the cell It is a glycoprotein present in 5 major forms (types I to V) All consist of three α-helical chains bound together They are similar in form and action but have specificity for different cell types Type I and III are involved in attachment of fibroblastic and endothelial cells (Tseng *et al* , 1981) Type II is involved in the attachment of chondrocytes (Kidwell *et al* , 1984) Type IV is involved in the binding of epithelial carcinoma cells (Palm and Furcht, 1982) Type V is involved in the binding of epidermal cells (Altalo *et al* , 1982) Collagen was shown to provide extended proliferation for mammary epithelia cells in SFM either exogenously or synthesised *in vitro* (Kidwell *et al* , 1982) Factors which stimulate collagen production include EGF, PGE_1, FGF, glucocorticoids and insulin-like peptides (Salomon *et al* , 1981) It can be used in the native form or can be denatured to form a gel which acts as a precoat for cells Native collagen has been used to form beads and act as microcarriers for suspending cells in solution (Yang *et al* , 1982) Type IV is probably the most common collagen used in SFM

1.2.2.2 Fibronectin

Fibronectin is also a basement membrane protein consisting of a dimer of α and ß chains of unequal length, linked together at the carboxyl end by disulphide bonds It is a glycoprotein (5-10% carbohydrate) of 550kDa molecular weight and is principally derived from plasma Structural variations exist depending on the source (due to alternative mRNA splicing) It is probably the most used adhesive factor in cell biology (Ruoslahti, 1988, Barnes *et al* , 1983, Burrill *et al* , 1981) It contains binding domains for fibrin, heparin, collagen, the cell (*via* RGD sites) and also to a DNA-binding domain

1.2.2.3 Laminin

Laminin is a large molecular weight (10^6) glycoprotein found exclusively in the basement membrane *in vivo* (Timpl *et al* , 1979) It consists of four subunits, bound by disulphide bonds which form a cruciform shape There is one α chain (400kDa) and three ß chains (200kDa) The short arms are involved in cell binding, probably *via* the two globular domains It has been shown to promote binding of epithelial cells PAM 212 to collagen type IV (Terranova *et al* 1980), hepatocytes (Carlsson *et al* 1981), interstitial epithelial cells (Burrill *et al* 1981) and Schwann cells (Palm and Furcht, 1982) Very little is found in serum It forms large polymers in the basement membrane and the ability to polymerise is dependent on the presence of divalent cations Laminin binds collagen type IV, heparin and proteoglycans

1.2.2.4 Other Attachment Factors

Chondronectin is a glycoprotein (180kDa) which is produced by chondrocytes and is found in the pericellular network (Hewitt *et al* , 1982) Its binding capacity is enhanced by collagen type II and proteoglycans It is found in the serum at levels of 1-20μg/ml

Other factors include Epibolin (65kDa) which provides attachment for epidermal cells Fetuin is a major protein of FCS and it too has adhesive functions There is also Vitronectin (serum spreading factor) which is found in the human serum, amniotic fluid and urinary proteins (Shaffer *et al* 1984) This has very similar activity to fibronectin and binds glycosylaminoglycans and heparin.

Proteoglycans contain a protein core to which glycosaminoglycan is added *e g* chondroitin sulphate, dermatin sulphate, keratin sulphate and heparan sulphate These interact weakly with laminin, fibronectin and collagen type IV

For anchorage-independent cells, no attachment factors are required for growth in suspension *In vivo*, however, for tumour cells to be metastatic, adhesion factors are needed in order for cells to leave the site of origin and travel through the blood stream to a secondary target site Metastatic tumours have been shown to use the above factors and some even produce their own adhesive factors (Liotha *et al* , 1986, Pande and Khur, 1988, David *et al* , 1994) The human squamous carcinoma cells produce thrombospondin (Varani *et al* 1986)

1.2.3 Vitamins

Vitamins are required for proper cellular functioning by acting as co-factors and co-enzymes in metabolic reactions They are components in all basal cell culture media (mostly B vitamins) The levels of vitamins may not be optimal for all cell lines, for example, Matsuya and Yamane (1986) found addition of Vitamin B_{12} to be necessary to supplement growth of several mouse cell types at low cell densities, while combination of choline and myo-inositol were found to improve the growth of human epithelial keratinocytes (Gordon *et al*, 1988) In addition to low cell densities, vitamin limitation may affect cell growth at high cell densities (Evans and Williams, 1988) Due to instability, some water soluble vitamin groups B and C had to be added to ATCC medium during the culture of CHO cells in SFM (Kurano *et al* , 1990)

Although vitamin C and E (α-tocopherol) have been used as antioxidants (Barnes, 1987), they are not always growth stimulatory Vitamin C was found to decrease the cell proliferation of post-embryo growth plate chondrocytes (Hennig *et al* , 1989) but increase arachidonic acid uptake, PGE_2 production and increase adenylate cyclase activity in B16 melanoma and non-malignant LLCMK cells (Stoll *et al* , 1994) Vitamin E was found to be inhibitory alone for rat mammary tumour line 64-24 cells but was necessary for growth stimulation with a lipid complex containing sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and phosphoethanolamine (Van der Haegan *et al* , 1989), probably by acting as an antioxidant It also inhibited the stimulation of *c-fos* mRNA levels in MCF-7 cells induced by IGF-I (Li *et al* , 1994)

Vitamin A has been found to induce differentiation of neuroblastoma (Ueno *et al* , 1993) and T47D cells (Thiele *et al* , 1988) but to prevent differentiation of lung carcinomas (Miyazaki *et al* , 1984) Combinations of vitamins A and E in SFM with Na_2Se0_3 or vitamin C were found to prolong the growth of primary cultures of adrenocortical cells (Simonian, 1982) and hepatocytes (Miyazaki *et al* , 1991) respectively Vitamin A alone inhibited TGF-ß stimulation of human mammary carcinoma cells (Halter *et al* , 1993)

Vitamin D was found to be inhibitory for a number of cell lines including human keratinocytes (Kobayashi *et al* , 1993), human leukemic cells (Reitma *et al* , 1983) and a breast cancer cell line (Eisman *et al* , 1989), but stimulated DNA synthesis in alveolar type-II cells (Edelson *et al* , 1994) Vitamin D was also found to reduce tissue plasminogen activator stimulated production of IL-6 and IL-8 in human fibroblast cell lines (Srivastava *et al* , 1994)

1.2.4 Trace Elements

Trace elements regularly used in SFM include Cu, Sn, Co Mn, Mo, Va, Ni, Zn and Se It has been found that by designing a SFM with a greater variety of trace elements, the requirement for albumin, insulin and transferrin could be eliminated (Cleveland *et al* , 1983, Darfler, 1990) Selenium is an essential trace element for many mammalian cells (Ganther *et al* , 1976, Iscove, 1984) Inclusion of selenium in media may be beneficial because of its ability to prevent the oxidation of lipids by functioning as a component of systems involving gluthatione peroxidase and superoxide dismutase (Hewlett, 1991) Indeed, Hatfield *et al* (1991) found transfer of cells from a selenium-free to a selenium containing medium resulted in an increase in selenocysteine

$tRNA^{[Ser]Sec}$ (initially aminoacylates with serine and the serine moiety is subsequently converted to selenocysteine, therefore designated as $tRNA^{[Ser]Sec}$) which controls the transcription and translation of glutathione peroxidase Growth stimulation effects of selenium have also been reported (McKeehan and Ham, 1976, Peehl and Stamey, 1986, Zhu *et al* , 1992), probably through its protection from oxidative radicals Iron is another trace element that may be added to serum-free medium to replace transferrin (see section 1 6) Calcium levels not only affect attachment and act as a co-factor for many enzymes, but have also been reported to enhance cell growth of some cell lines in SFM (Praeger and Cristofalo, 1986, Robinson and Wu, 1991)

1.2.5 Lipids

Serum contains two classes of lipid transport proteins and their lipid components albumin, which carries free fatty acids, and lipoproteins, which carry phospholipids, triglycerides and cholesterol Although lipid requirements vary for different cell types, it is more economical for cells to use intermediates of lipid metabolism, (*e g* cholesterol, long-chain fatty acids and glycerides), than to start from scratch, provided that the cells are not auxotrophic for a particular lipid Lipid requirements and especially fatty acid requirements are traditionally supplied by using albumin as the lipid carrier Albumin substitutes include polyethylene glycol (PEG), carboxymethyl cellulose (Blasey and Winzer, 1989, Shintani *et al* , 1988), liposomes (Darfler, 1990), and α and ß cyclodextrins (Ohmori, 1988)

Cholesterol is a requirement for all growing and dividing cells as it is the main component of the cellular membrane, precursors for prostaglandin synthesis and is an alternative source of energy (Kan and Yamane, 1982, Saier, 1984, Sato *et al* , 1987) Many cells can synthesise cholesterol but some cells are auxotrophic and require exogenous addition (Chen and Kandutsch, 1983) Cholesterol alone can have a negative effect on cell proliferation (Buntemeyer *et al* , 1993)

Free fatty acids are essential nutrients for other cells, especially linoleic acid, arachidonate, palmitic and oleic acid (Ham, 1963, Spieker-Polet and Polet, 1981)

Lipoproteins are required by some cells in SFM (Tauber *et al* , 1981, Gospodarowicz and Cheng, 1987) For human arterial smooth muscle and lung fibroblasts, lipoproteins were found to be primary mitogens and growth promoters while cholesterol or essential fatty acids were not

(Bjorkerud and Bjorkerud, 1994) Not only is it sometimes necessary for growth but HDL was found to enhance the production of IL-1 in A431 human keratinocytes (Blasey and Winzer, 1989) Phospholipids were found to have a stimulatory effect on MDCK and normal kidney cells in SFM by interacting synergistically with insulin (Bashir *et al* , 1992)

A variety of commercially available lipoprotein complexes can be used to support the growth of a variety of cells without having to worry about solubility, *e g* Ex-cyte Ex-cyte encompasses a variety of lipoprotein mixtures which haven been found not only to promote growth but also to improve metabolite production (Blasey and Winzer, 1989, Hewlett *et al* , 1989, Belisle *et al* , 1990)

1.3 INDUSTRIAL OVERVIEW

There is huge potential for the use of mammalian cell culture in the production of a wide range of commercially valuable products for the veterinary and pharmaceutical industries (Spier and Griffiths, 1988) Much progress over the past 15-20 years has occurred in the development of systems for growing anchorage-dependent and anchorage-independent mammalian cells for example, in the use of large-scale computer controlled bioreactors (as large as 10,000L) and high density systems which can maintain cell densities of 10^8 cells/ml for long periods (for references see Griffiths, 1992) Improvements and advances have been made in the developments of new media for growth and production and in advances in genetic engineering, both of which, have not only increased the range of products but also the yield The improvements in media optimization and increased yields have considerably reduced the advantage that bacterial systems had over mammalian cells in terms of productivity and cost

Mammalian cell products include vaccines, monoclonal antibodies (mAbs), interferons, other lymphokines, urokinase, tissue plasminogen activator and human fertility hormones Two traditional methods of production exist Monoclonal antibody production by hybridomas and myelomas and expression of recombinant gene products from genetically engineered cells

Typically there are two categories of media used in large-scale fermentations, the growth medium and the production medium The growth medium usually contains 5-15% serum while the production medium contains a much smaller amount (less than 5% serum) Some systems have taken advantage of this by using a serum-supplemented medium for the growth phase, followed by the introduction of a SFM for the production phase In designing a SFM, the requirements for growth and production may vary

The cost of the basal medium is about the same as that for a microbial system, but the addition of various supplements (serum and antibiotics) make the medium up to 15 times more expensive (Griffiths, 1986) So although there are advantages to the large-scale use of SFM, the cost must be of high priority so that manufacture can be as economic as possible In addition to labour and time being required for the transfer from the growth to the production medium, there is also the increased risk of contamination It has also been reported that serum seemed to inhibit mAb production in comparison to SFM (Tharakan *et al* , 1986)

In addition to the cost, the purity of products obtained from mammalian cells growing in serum-free medium is important, since the quality of pharmacological products could be significantly affected by minute amounts of serum components International regulations for good manufacturing procedures (GMP) restrict the amount of serum constituents in the final product when making injectable vaccine products as allergic reactions could be induced

1.3.1 Monoclonal Antibody Production

One of the biggest successes for SFM has been in the production of monoclonal antibodies (mAbs) Immunoglobulins (Igs) are in great demand due to their medical, clinical and analytical applications For a large variety of the hybridomas grown in SFM, there is little variation in the medium composition Only four basal media are extensively used DMEM, Ham's F-12, RPMI-1640 and IMDM The supplements are almost always insulin, transferrin and ethanolamine (Murakami *et al* , 1982) In many cases BSA is included also For spinner cultures, phosphoethanolamine or phosphatidylglycerol may also be added Kawamoto *et al* (1983b) suggested that some hybridomas required low density-lipoproteins or fatty acids such as oleic and linoleic acid, however, the requirement for lipids is not universal (Chang *et al* ,1980) These hybridomas are not fastidious and so SFM are relatively cheap in comparison to SFM designed for growth of anchorage-dependent cells (few growth factors and no attachment factors)

Not only has it been possible to grow hybridoma cells in SFM, but low protein (Blasey and Winzer, 1989, Jager *et al* , 1988) and protein-free (Darfler, 1990) media have also been developed to maximise production (equivalent titres of mAbs seen in SFM and SSM) and minimise downstream processing costs In addition there may be fewer problems with Federal Drug Agency (FDA) approval and less potential for carry-over of adventituous agents In some situations, the use of a protein-free medium is not viable Chua *et al* (1994) reported that for the hybridoma cell line 2HG11, IgG production in the basal medium eRDF (RPMI DME Ham's F12 in 2 1 1 enhanced with glucose, amino acids and vitamins) was similar in serum-free and serum-supplemented media but that removal of BSA from the SFM resulted in a much lower titre of IgG Ozturk and Polsson (1990) also reported similar findings

Optimization of production is of prime importance *In vivo*, one plasma B cell in the lymph nodes secretes about 2,000 antibodies per second (McKeehan *et al* , 1990) This is equivalent

to 1 7x10^8 molecules or 43pg/cell/day *In vitro* however, at 10^6 cells/ml only 1 gram of IgG is produced by 10^{11} cells/day *i e* 10pg/cell/day It is obvious that many of the systems used are not optimized for the production of antibodies

Optimization of medium usage has led to the development of high cell densities Namalwa, a human lymphoblastoid cell line has been grown in SFM in perfusion cultures (cell densities higher than 10^7 cells/ml) to produce human ß-interferon and human lymphotoxin (Miyaji *et al* , 1990a,b,c, Hosoi *et al* , 1991) Human hybridomas were grown in SFM to produce anti-tetanus toxoid and anti-HBsAg human mAbs (Kitano *et al* , 1991) However, this SFM required the addition of a stimulatory fraction from adult bovine serum or a combination of polyethylene glycol and swine LDL to stimulate good growth and mAb production

1.3.2 Expression of recombinant proteins

Bacterial and yeast expression systems have been used successfully to synthesise a variety of recombinant gene products However, post-translational modification in higher eukaryotes is more complex than in lower eukaryotes counter parts So where post-translational modification (*e g* glycosylation) is necessary for proper biological activity of the product, mammalian systems are often the preferred choice (Goto *et al* , 1988) Animal cells provide an ideal environment in which recombinant proteins can be properly transcribed and glycosylated A variety of cell lines including CHO, Vero, CV-1, Namalwa (lymphoblastoid) and C127 cells have been found to be suitable recipient mammalian hosts to accommodate transfection and expression of a variety of recombinant gene products These products include interferon (Chernajovsky *et al* , 1984, Mitrani-Rosenbaum *et al* , 1984), tissue plasminogen activator (Kaufman *et al* , 1983), growth hormone (Pavlakis and Hamer, 1983), polio vaccine (Larsson and Litwin, 1987), rabies vaccine and hepatitis B antigen (Michel *et al* , 1985, Christman *et al* , 1982), erythropoietin and interleukin-2 (Conradt *et al* , 1986), human renin (Asselbergs *et al* , 1994), and α-1-antitrypsin (Paterson *et al* , 1994)

Large-scale cultivation of anchorage-dependent cells usually involves the use of microcarriers for adherence Satoh *et al* (1991) carried out extensive research to design a suitable large-scale SF cultivation of TRC-29SF (human renal carcinoma) to produce human macrophage colony stimulating factor (M-CSF) The M-CSF production was improved by gene amplification with dhfr-MTX system (Satoh *et al* , 1991) The cells were grown in SFM in a perfusion culture on microcarriers with a final cell density of greater than 10^7cells/ml

1.3.3 Virus Production

The development of serum-free nutrient media for the production of human and veterinary viruses is not as simple as that seen in the production of monoclonal antibodies Many of the cells used in viral production are anchorage-dependent

Although the absence of adventitious (especially viral) contaminants and antibodies is of prime importance, the prohibitive cost and the regulatory surveillance of the industry restricts the broad adoption of serum-free media FCS has been replaced by cheaper sources of serum (*e g* bovine and equine) which are usually chemically treated or gamma-irradiated to destroy any possible viral contamination Commercially available SFM like OptiMEM™ is a nutrient-enriched medium which has been found to support virus production in WI-38, MCR-5, IMR-90, Vero, MDBK, BHK and other primary and established cell lines (Jayme, 1991) Human immunodeficency virus production has been successfully carried out in HUT 78 cells in a serum-free medium (AIM-V)

Mention should also be given to the use of insect cell cultures in the large-scale production of medical, diagnostic and veterinary products The baculovirus expression system has been used in insect cells such as *Spodopteran frugiperda* (SF9 or SF21) cells
A large number of recombinant products have been produced *e g* α-interferon (Maeda *et al* , 1985), hepatitus B surface antigen (Kang *et al* , 1987), lymphocytic choriomeningitis virus G protein (Matsuura *et al* , 1987), interleukin 2 (Smith *et al* , 1985), thrombomodulin (Ogata *et al* , 1993), and interferon-ß (Smith *et al* , 1983) The ability of these cells to grow well on a large-scale (Murhammer and Goochee, 1988, Maiorella *et al* , 1988) and in serum-free medium (Vaughn and Fan, 1989, Hink, 1991) offers a comparatively inexpensive system of production However, although invertebrate cells have a greater capacity for post-translational modification than microbial systems, very complex glycosylations which are sometimes necessary for biological activity, cannot be carried out

1.3.4 Economic feasibility of SFM on large-scale

As mentioned earlier, the use of serum in large-scale processes can be costly Incorporation of the high cost of growth factors and attachment factors together with the preparation time it requires to make up these often complicated media, may make the SFM as expensive or more expensive than serum-supplemented media

To overcome the expense, less fastidious cells should be chosen This can be seen with the myelomas and hybridomas used in the large-scale production of monoclonal antibodies They have no requirement for attachment factors and little requirement for expensive growth supplements

Optimization of the medium is also a way of cutting production costs A knowledge of the growth requirements of the cells can be used to optimize basal media For example depletion of amino acids correlated to a decrease in culture viability and biological productivity (Luan *et al* , 1987a, Jager *et al* , 1988) By incorporating or adding more amino acids, growth and production can be continued without a significant increase in cost The rapid breakdown of glucose to lactic acid can be reduced by the partial replacement of glucose by other hexoses (*e g* mannose) This has helped to maintain growth and productivity in high density cultures

Immobilization of growth factors to a membrane surface has allowed for the development of alternative 'protein-free' media Liu *et al* (1993) coimmobilized insulin and collagen on the surface of a hydrolysed polymer membrane to support the growth of anchorage-dependent cells (STO and 3T3-LI) and a fibroic sarcoma The cell growth was found to be accelerated more strongly by immobilized than by free proteins The membrane could be repeatedly used up to 12 times without a significant loss in activity By immobilising growth factors or hormones on a membrane, they do not interfere with downstream processing This method would be ideally suited for use in a perfusion system where the expensive proteins could be immobilized on a membrane and an inexpensive protein-free medium could be used to perfuse the cells

The formation of ammonia from the deamination of glutamine can prematurely induce cell senescence Replacement of glutamine by a di-peptide, glycyl-L-glutamine (Jayme, 1991) resulted in reduced ammonia build up and enhanced mAb production Geaugey *et al* (1989) found that enrichment of hybridoma medium with glutamic acid during the culture period improved cell viability and mAb production However, Schlaeger *et al* (1994) reported that when dipeptide L-alanyl-L-glutamine replaced glutamine, there was a decrease in ammonium production but also a decrease of more than 30% in the production of thyroid stimulating hormone antibody, with only a shght reduction in cell density It was further suggested that glutamine appeared to be the critical nutrient due to high accumulation of ammonia

For mAb production there is also the potential to use immunoglobulin production stimulating factors (IPSF) Additives which have been found to contain IPSF include chicken egg yolk lipoprotein, Royal jelly and casein However, most IPSFs so far reported, stimulate IgM production predominately (Murakami *et al* , 1991)

There are many commercial products for growing such cells in serum-free medium Aside from the proprietary retention of information on some of the factors in the SFM, most of these products are suitable for small-scale to moderate-scale use The cost may be prohibitive for large-scale productions Griffith (1986) reported that medium costs using FCS were £700 per 40L fermentation, while the cost of SFM were much more expensive, about £600 per litre This SFM included EGF, FGF, PDGF, transferrin and fibronectin Obviously, the cost will depend on the requirements of the cells under serum-free conditions For CHOK1 cells, the SFM designed by Mendiaz et al (1986), cost around £350 per 40L while serum at a concentration of 5% vol/vol cost between £320 - £400, depending on the quality of the serum (costs determined on 1994 Sterling prices and not including the basal medium)

The use of high density cultures has also been used to reduce the cost of medium High densities have been attained by use of encapsulation techniques and growth of cells m hollow fibre and packed bed fermentations Shirai *et al* (1989) immobilized cells in calcium alginate beads m a SFM Recombinant von Willebrand factor was produced by CHO cells cultured in macroporous microcarriers in SFM (Mignot *et al* , 1990) Liu *et al* (1991) used a large-scale hollow fibre bioreactor for the long-term cultivation of HepG2 cells which produced a crude conditioned medium protein Production of this factor was as good when the cells were grown m serum-free as in serum-supplemented medium

Bliem *et al* (1990) used a packed bed reactor for the mAb production under serum-free and protein-free conditions Over a 4 week period over 110g of antibody was produced under serum-free conditions but only 25% of the activity was seen under protem-free conditions On transfer from serum-free to protein-free conditions, the drop in antibody production occurred with a drop in metabolic activity Cells initially grew in SFM (insulin, transferrin and albumin in RPMI/DME medium) When the SFM was replaced with a basal medium (RPMI/DME), a dramatic drop in growth and metabolic activity was seen When a combination of Coon's F12/DME was added, 25% of the antibody producing activity was retained As these cells had not adapted to growth in protein-free medium, it may be that some factor required to stimulate

the production of antibodies was absent or limiting in the two combinations of basal media

Recently, Lee and Palsson (1993) reported that entrapment of cells in calcium alginate beads (in theory any other form of entrapment) prevented a non-producing population of hybridomas from taking over the producing population by limiting the growth of the non-producing population This led to a stability in monoclonal antibody production

Another possibility to reduce costs is the extractions of multiple products from the same broth and the use of high cell densities *e g* perfused systems In a closed perfused system, 3-fold to 4-fold savings could be made on medium (Griffiths *et al* , 1982) However, this would mean increased downstream processing problems in order to isolate each individual factor and would probably yield a smaller quantity of each product

1.3.5 Problems with using SFM on a large-scale

When a cell line is used in a production process, not only should the cell grow in the SFM but it would be necessary to test the cells on a technical scale equivalent to the final production process in order to ensure the performance of the cells is not suffering from mechanical shearing It may also be necessary to examine the character of the product itself as Moellering *et al* (1990) suggested that the composition of the medium could affect the physicochemical properties of the products The biochemical properties of some cell lines do remain the same *e g* , GH3 rat pituitary carcinoma cells secrete both prolactin and growth hormone in culture medium Production of prolactin in SFM is about 70% of that seen in serum-supplemented medium (Wu and Sato, 1978) On the other hand, Chang *et al* (1980) grew a number of hybridomas in SFM and found that one of the adapted hybridomas was able to secrete for more than 3 months whereas the parental myeloma could only secrete for 6 days

In addition, factors that protect the cells from shearing effect may also need to be incorporated (Papoutsakis, 1991), for example Pluromc F68 (Marquis *et al* , 1989), a co-polymer of polyoxyethylene and polyoxypropylene or polyvinylpyrrolidone (Gurhan and Ozdural, 1990)

Another problem with SFM is its stability Due to the requirement for hormones and growth factors, many SFM have a limited shelf-life and also require much time and manpower to produce An ideal SFM product should be easily made up, preferably autoclavable and have

a stable shelf-life of at least 2-3 months To this end, the development and use of synthetic compounds which can replace the more time and temperature labile proteins is of great interest Minamoto *et al* , (1991) used Gly-L-Glutamine and L-Ala-L-Glutamine to replace the L-Glutamine usually used in culture media These compounds could withstand autoclaving and were stable on storage at 37°C for 4 months More importantly, they were effective on cells in culture α-Cyclodextrin was substituted for BSA and Fe-gluconate could replace transferrin Insulin was found to be stable in the presence of Fe-gluconate Such a SFM has obvious advantages over use of serum

1.4 COMMERCIALLY AVAILABLE SFM

Commercially available SFM are now produced by companies including Sigma, Boehringer Mannheim, Costar, Flow Laboratories, Hyclone, New Brunswick and many more The result is a large selection of commercially available serum-free media which are designed for specific cell lines Table 1 4 shows the variety of commercially available SFM supplements complete media that are available

Three groups of commercially available SFM medium exist

1 SFM additives These are designed to be used instead of serum with the basal medium of choice These 'SFM additives' are not as such serum-free, but do have some advantages over traditionally serum-supplemented medium including lower protein and lower immunoglobulin content These SF additives are not as selective as traditional SFM and may be used for a wide variety of cell types However, the additives consist of fractionated or modified serum components (for example, CPSR, and LPSR from Sigma)

2 Serum-free supplements These usually consist of a combination of the more commonly used SF components like insulin, transferrin, selenium, growth factors, hormones and trace elements They are usually used in combination with ATCC (1 1 v/v Dulbeccos MEM Ham's F12) as the basal medium

3 Completely defined SFM These media are usually optimized for the growth of a specific cell line or cell type Increasing the reliance on the ability of the basal medium to supply nutrients and/or trace elements has been investigated in order to eliminate the requirement for serum. This means that little or no additives like growth factors or hormones are needed to be

Table 1.4 Commercially available SFM

PRODUCT	COMPANY	CELL LINE	COMPONENTS
SF-1 supplement	Costar	B and T Cells, rat/mouse hybridomas m/m hybridomas	IMDM + BSA, EA, Ins, Tf, LA, palmitic acid and oleic acid
SF-X	Costar	Anchorage-dependent cells and B Cells	As above + Trace elements($MnSO_4$, SeO_3, $(NH_4)_6Mo_7O_{24}$, $NaVO_3$, $NiSO_4$, $SnCl_2$, $AlCl_3$, $AgNO_3$, $Ba(C_2H_3,O_2)_2$, KBr, Cd_3Cl_2, $CoCl_2$, $Cr(SO_4)_3$, NaF, GeO_2, Kl, RbCl and $ZrOCl_2$
MCA 1	Costar	Hybridomas (x-63, NS-1, Sp2/0, Y-3)	IMDM + Tf, Ins, ßEs, EA, BSA, ESG, AA, non saturated fatty acids
Profree LC-115	Marcor	Plasmocytomas, hybridomas, myelomas	Inorganic salts, glucose, HEPES, LA, Lipoic acid, hypozanthine, Na pyruvate, thymidine, 21 amino acids, 11 B vitamins and 18 trace elements TI required for anchorage-dependent cells
Biorich 1	Flow	Anchorage-dependent cells and hybridomas	ATCC + trace elements
Biorich 2	Flow	Myelomas, hybridomas and lymphoblastoid cells	'Greater than 60 high quality chemicals including pyruvate, BSA and Ins'
Nissui SFM-101	WBAG	Mouse Hybridomas P3-U1, NS-1, Sp2/0, Ag8-653 and Namalwa	Basal medium + Ins and Tf
G I T Medium	Wako Pure Chemicals	Anchorage-dependent and independent cells HeLa, NS-1, HL-60,K562, BHK-21, CHOK1, MRC-5,Vero	GFs (3mg/ml), Ins, Tf, EA, $NaSeO_3$, LA trace elements
ITS+	Flow	many cell types	ATCC + Ins, Tf, Se, LA + BSA
UltroSer G	IBF Biotechniques	Hep, BHK, Hela, MRC-5, Vero and CHO	Growth factors, Adhesive factors, mineral trace elements, hormones Transport proteins, vitamins and aprotinin
UltroSer HY	IBF Biotechniques	hybridoma and myelomas Sp2/0, P3, HL-60, IH8-4	BSA, lipids, Tf, Ins, Vitamin C and B_{12}, $NaSe_2O_3$
Nutridoma SP	Boehringer Mannheim	murine myelomas and hybridomas from Sp2/0	Serum albumin, Tf, Ins, other specific inorganic and organic molecules
Bio-MDM-1	Biological Industries	Anchorage-dependent cells	Insulin
Soft cell	TCS	Anchorage-dependent and independent cells	Ins, Tf, testosterone, EA, a variety of saturated and unsaturated fatty acids
CSPR	Sigma	most cell types	serum/ treated
LPSR	Sigma	most cell types	contains growth enhancement components and carrier proteins
CGA™	Otisville Biopharm	most cell types	fractionated bovine serum, low protein

Table 1.4 continued

PRODUCT	ADVANTAGES	DRAW BACKS	STORAGE
SF-1 supplement	No IgG	requires adaption + ESG	4°C for 2 years lyophilized
SF-X	Any basal medium	may require adaption + ESG	4°C for 2 years lyophilized
MCA 1	only 5µg/ml IgG	requires serum for some cell types contains ESG	2-8°C for 1 year
Profree LC-115	long-term cultivation of transformed cells effective on some primary cell lines	require large cell densities and much feeding for some primary cells may require adaption TI required for anchorage-dependent growth	
Biorich 1		Adaption required	2-8°C for 1 year
Biorich 2	no supplements required	mAb production affected	2-8°C for 1 year
Nissui SFM-101	low protein(20µg/ml) good for mAb production comparable to serum hybridoma selection without HAT-medium	No description of medium components	2-5°C in dark for 1 year
G I T Medium	Wide variety of cells no preculture no bovine-derived antibodies	Based on Gfs isolated from serum (3mg/ml)	0-10°C for 6 months -20°C for longer
ITS+			
UltroSer G	no IgG, low protein, good for growth and production	may require adaption	4°C for more than 18 months
Nutridoma SP	Flexibility with basal medium low protein	contains human- or bovine-derived proteins adaption required	Stable for 1 year at room temperature
Nutridoma NS	flexibility with basal media low protein	may require adaption	Stable for 1 year at room temperature
Bio-MDM-1	low protein (30µg/ml)	requires attachment factor and sometimes hormonal addition	4°C for 6 months without much light
Soft Cell	low protein growth and production of mAbs	may require adaption higher cell densities favoured	4°C for 1 year
CSPR/LPSR/CGA™	low protein/IgG	serum derived contents	as for serum

Abbreviations Ins = insulin, Tf = transferrin, m/m = mouse/mouse, EA = ethanolamine, ß-Es = ß-Estradiol, LA = linoleic acid, ESG = Ewings Sarcoma growth factor, AA = amino acid, TI = trypsin inhibitor, mAb = Monoclonal antibody, HAT = hypoxanthine-aminopterin-thyroxine

incorporated into the medium for growth (Darfler, 1990) Ham's F12 was originally developed for the growth of CHO cells in SFM on this basis Commercially available SFM like the MCDB series have been developed for use with a variety of cell lines

Another example is the series of Ultroser products, which have been used for example in growth and prostaglandin production in mouse embryo fibroblasts (Durant *et al* , 1989) These formulations normally contain quite a variety of trace elements, ammo acids and vitamins in addition to the factors used in SFM supplements However, a problem with some of these SFM is that the exact composition is not made available to the user

In addition to the advantages of working in a more defined environment, the intensive work involved in designing and producing SFM is gone For companies, the specificity of SFM for certain cell lines means that there are plenty of niches for product expansion

Commercial SFM alternatives have disadvantages Many cells need a period of adaption to the SFM The higher the purity of the product, the more expensive to SFM Some companies do not divulge information on the full composition of the SFM Recently, a complete cell culture system was made available Epipack^{R} and Bronchialpack are supplied by Clonetics, contain SFM and the adapted cells *e g* normal human keratmocytes and bronchial/tracheal epithelial cells respectively

1.5 THE ROLE OF INSULIN IN SERUM-FREE MEDIUM

Insulin or insulin-like growth factor I (IGF-I) is required for growth by virtually all cell types In many SFM, insuhn is used at superphysiological levels (1-20μg/ml) At such high concentrations mitogenic activity is observed In many cases it was found that IGF-I or IGF-II could replace insulin at normal physiological concentrations (ng/ml) The possibility of using IGFs instead of insuhn would mean a reduction in the protein concentration of SFM In addition, replacing bovine-derived insulin with recombinant insulin or recombinant IGFs would lead to a more defined medium The question was then asked, does msulin have mitogenic activity of its own or is the mitogenic effect observed because msuhn mediates DNA synthesis through the IGF receptors? To answer this question, a knowledge about the structure and functions of insuhn and the IGFs is required

Insulin, IGF-I and IGF-II are members of a class of growth factors Although there is considerable homology among the three proteins, great variability exists in their biological effect (both metabolic and mitogenic) Insulin and IGF-I are involved in ammo acid uptake, control of fatty acid metabolism and DNA synthesis for many cell lines (Pledger *et al* , 1978, Cherington and Purdee, 1980, Hoffmann *et al* , 1989, Conover *et al* , 1985) The function of IGF-II is less well understood, although its mitogenic activity is usually associated with cells at a fetal stage of development

1.5.1 Structure of Insulin, IGF-I and II

Insulin and the IGFs are single-gene products from separate genes (Ullrich *et al* , 1985) Expression of different transcripts occur due to differences in exon splicing between species, different transcription start sites (Simmons *et al* ,1993) or incomplete processing or truncation at one of the termini (Ogasawara *et al* , 1989)

Insuhn is a polypeptide (Mw of about 5,700) with two subunits, while IGF-I and II are single polypeptides (Mw of about 7,500) The three show considerable amino acid homology, with strict conservation of the structurally important amino acids such as cysteine for intra-chain disulphide bridges, and glycine in the hydrophobic core This ensures that the tertiary structures are very similar All are synthesized as pro-proteins and cleaved to yield the active protein The source and secretion of these polypeptides *in vivo* are varied Insulin is secreted by the β cells in the pancreas and this secretion is controlled/affected by physiological glucose

and amino acid levels IGF-I is secreted from liver cells into the blood, where it acts as an endocrine hormone, mediating the anabolic actions of the pituitary growth hormone *In vivo*, its production is controlled from the pituitary by feed back inhibition IGF-I is also secreted by a variety of other cell types and tissues, where it can act as a paracrine or autocrine growth factor locally IGF-binding proteins are used to control the activity between source and the site of action (Clemmons, 1993)

IGFs are major tumour growth factors whose autocrine activity has been associated with lung, breast and gut (Jing *et al* , 1991, Ohmura *et al* , 1990, Chung and Antomades, 1992, Favoni *et al* , 1994) In the lung, a combination of overproduction of IGFs and low levels of IGF-binding proteins, results in uncontrolled growth In the breast, increases in receptor number or receptor sensitivity, make the cells more sensitive to IGFs

The source and secretion of IGF-II is most commonly associated with the fetal stage of development (Powellbraxton *et al* , 1993) Some transformed cells also produce IGF-II-like autocrine and paracrine factors *e g* neuroblastoma BE(2)-C cells (El-Bradry *et al* , 1991)

Other growth factors and hormones have profound effects on the mode and the extent of activity of insulin and the IGFs For example, when Balb/c-3T3 cells are grown in serum-free medium IGF-I acts as a progression factor taking competent cells through the G_1 phase (Pledger *et al* , 1978) However to make the cells competent PDGF or EGF was also required (Stiles, 1983) This has been reported for many other cell types (Alkhalof *et al* , 1991, Van der Burg *et al* , 1988) TNF-α was found to suppress tyrosine phosphorylation of the ß-subunit of the insulin receptor and the insulin receptor substrate (IRS-I) (one of the post-membrane proteins involved in signal transduction of the insulin signal) protein in Fao cells (Feinstein *et al* , 1993) Allen and Boxhorn (1989) found that IGF-I caused minimal proliferation but stimulated differentiation in skeletal muscle satellite cells

1.5.2 Receptor Binding

Receptor binding studies have revealed three distinct receptors based on their affinity for insulin and the IGFs Each has high specific activity for its own receptor with a low cross reactivity for the other receptors Insulin and IGF-I receptors represent a subclass of the receptor tyrosine kinases Investigations have revealed sequence homologies to human EGF receptor and the

members of the src family of oncogene products (Ullrich *et al* , 1985) Insulin and IGF-I receptors are present in nearly all tissues IGF-II receptors were originally thought to have a similar structure to the mannose-6-phosphate (M6P) receptor, but was recently shown to be the same receptor The IGF-II/M6P receptor exhibits no tyrosine kinase activity

The insulin receptor (IR) has been extensively studied and the postulated model consists of an immunoglobulin-like structure (ß-S-S-α)-S-S-(α-S-S-ß) in which α and ß are glycoprotein subunits with apparent molecular weight of 125,000 and 90,000 respectively The receptor components are derived from a single glycosylated precursor The structure is symmetrical with the α-subunit as the extracellular component and the ß-subunit comprising the transmembrane and cytoplasmic domains Subsequent studies revealed that the IGF-I receptor was structurally homologous to the Insulin receptor (for references see Czech, 1982, 1988, 1989) with the same molecular weight and physical properties A maximum homology of 85% is found in the tyrosine kinase domain (Ullrich *et al* , 1985) The receptors, like the proteins are derived from individual genes on separate chromosomes

The IR acts hke an allosteric enzyme When msulin binds, a conformational change results in the α subunit This causes activation and autophosphorylation of tyrosine and serine residues on the ß-subunit This leads to the activation of tyrosine residues of several proteins including Insulin receptor substrate-1 (IRS-1) (Waters *et al* , 1993, Rose *et al* , 1994), MAP kinase (Microtubule associated protein kinase), phosphotidyhnositol-3-kinase, pp175 (insoluble cytoskeleton associated protein (McClain *et al* , 1990), pp60, pp120 (Najjar *et al* , 1993) and pp220 (Condorelli, 1989) and inositol nucleotide phospholipids (Gilmore, 1988) It is also believed that activation of normal p21-H Ras G-protein may be involved in signal transduction of insulin and IGF stimulation (Medema *et al* , 1993, Jhun *et al* , 1994) Beyond kinase activation, the signal transduction path is poorly understood Nuclear proteins called lamins and immediate early genes which encode the proto-oncogenes c-*jun* and c-*fos* are known to be associated with insulin stimulation (Ong *et al* , 1987, Chio and Chang, 1992)

Less reference is made to IGF-II in SFM The physiological role of IGF-II is as yet unclear but is usually associated with cells at the fetal stage of development (Ferguson *et al* , 1992) It has a higher affinity for the msulin receptor than IGF-I does (Czech, 1989) The IGF-II receptor is divergent from the msulin and IGF-I receptors in structure and signal transduction

It consists of a predominantly extracellular single poly-peptide with a molecular weight of approx 250,000 and shows no structural relationship with IGF-I It has a higher affinity for binding IGF-II than IGF-I and very little affinity for binding insulin with no apparent intrinsic tyrosine kinase activity but serves as a substrate for membrane associated tyrosine kinase activity Although the IGF-II and M6P share the same cell surface receptor, the M6P and IGF-II binding domain on the M6P receptor were found to be distinct (Dahms *et al* , 1994) In rats IGF-II is not solely associated with the fetal stage, IGF-II mRNAs were also found in adult tissue from the central nervous system (Lund *et al* ,1986)

Controversy over the mitogemc or other activities of IGF-II exists It is mitogenic for numerous cell types (Humbel, 1990) although most of the activity has been found to be through the IGF-I receptor (Casella *et al* , 1986, Czech, 1989) It can also act as an autocrine factor m cellular differentiation, as seen with myoblast differentiation (Florini *et al* , 1991) Others postulate that the activity of IGF-II is by its own receptor Murayama (1990) suggested that coupling of IGF-II to the IGF-II/M6P receptor stimulated a guanine nucleotide binding protein with an 40kDa α-subunit, G_{i2} protein which could provide a mechanism for signal transduction Human erythroleukemic cells were found to be stimulated by IGF-II through its own receptor (Hartmann, 1992)

1.5.3 Mechanisms of mitogenic action of Insulin

The mechanisms of mitogemc action of insulin vary from cell type to cell type Most studies show insulin as a mitogemc factor by mediating its effect through the IGF-I receptor

Studies of the overlapping functions of insulin and IGF receptors in 3T3-L1 adipocytes suggested that the α subunit of the insuhn and IGF-I receptors have equal potential to stimulate metabolic and mitogemc responses For the NIH-3T3 cells both insulin and IGF-I were able to elicit immediate response (glucose transport) but exerted different mitogenic signalling potentials (Lammers *et al* , 1989) Through the use of synthetic receptors they found that the differential effects of the two peptides were related to the cytoplasmic domain (ß subunit) of the receptor only Tartare *et al* (1994) suggested that the carboxyl (COOH) terminal domain of the insulin receptor was more tightly coupled to the stimulation of glycogen synthesis than the IGF-I receptor in NIH-3T3 cells, while both exhibited similar mitogemc activity MaGoffin and Erickson (1988), compared growth stimulating effect of various insulins and IGF-I on primary ovarian interstitial cells and found insulin was most effective at μg quantities while IGF-I was

as effective at ng quantities These results were also observed on fetal rat epithelial cell (Jassal *et al* , 1991) and oligodendrocyte development (McMorris *et al* , 1986), normal adult human keratinocytes (Neely *et al* , 1991), canine tracheal smooth muscle cells (Yang and Chou, 1993) and neuroblastoma (Meghani *et al* , 1993)

Verspohl *et al* (1988) suggested that differences in the biological activity of insulin and IGFs is more likely due to cellular distribution than to a fundamental difference in action For example, classical metabolic target tissues for insulin include fat and liver These cells have many insulin receptors and few IGF-I receptors, while fibroblasts have mostly IGF-I receptors

The mitogenic effects of insulin have been reported to be mediated through the IGF-I-R while the metabolic activities of IGF-I were mediated through the IR (Conover *et al* , 1989, Oonk *et al* , 1989) However McClain *et al* (1990) suggested that IGF-I could stimulate metabolic activities through its own receptor

Others have reported insulin as being effective at ng levels Nagarajan and Anderson (1982) found that insulin could stimulate growth of F9 embryonal carcinoma cells apparently through its own receptor Mamounas *et al* (1989), suggested that insulin acted through its own receptor at physiological levels to stimulate growth of CHO-K1 cells in SFM as insulin showed poor competition for the IGF-I receptor at these concentrations Affinity labelling showed the receptor to which insulin bound to be typical of the α-subunit of the insulin receptor

Loo *et al* (1990) reported that the growth response of serum-free mouse embryo cells to insulin and IGF-I was similar over a range of 0 001 - 1 0 μg/ml Rat hepatoma, pre-implantation mouse embryo (Harvey and Kaye, 1992) and porcine granulosa (Barano and Hammond, 1984) cell lines have been found similarly to use insulin at low physiological levels

Flier *et al* (1986) used an IGF-I receptor antibody αIR-3 to show that insulin acts at its own receptor to stimulate thymidine incorporation at concentrations of less than 1 μg/ml but at higher concentrations, the action is suggested to be mediated through the IGF receptor in human skin fibroblasts

Where insulin-stimulated mitogenic activity occurred at physiological concentrations, a variety of mechanisms are presented The ability of F-9 and SF mouse embryo cells may be related

to the fetal stage of development, at which stage, several investigators have found variations in the ß-subunit of the IGF-receptor For example, Van Obberghen-Schilling and Pouyssegur (1983) found two types of IGF receptors One was insulin sensitive (similar to IR) and other was insulin insensitive Jonas and Harrison (1985) also found two types of IGF-I receptors in placental cells Alexandrides and Smith (1989) found a novel IGF-I receptor in fetal muscle Both IGF-I and insulin could stimulate a mitogenic response at physiological concentrations in fetal muscle The novel fetal ß-receptor subunit (105kDa) was found to be more closely associated with the IGF-I than the insulin receptor After two weeks, decreased expression of a novel IGF-I receptor (not present in adult cells) resulted in reduced responsiveness to insulin

Mutations in the carboxyl region of the ß-subumt may also result in a mitogenic signal from insulin at low concentrations Takata *et al* (1992) characterized a mutant IR which had two tyrosines in the carboxyl-terminus replaced by phenylanaline Compared to the wild type, enhanced sensitivity to insulin stimulated DNA synthesis was seen without affecting glucose uptake Enhanced activity of the S6 kinase protein also occurred Thies *et al* (1989) observed similar changes in carboxyl terminal deletions in Rat-1 fibroblasts It was proposed that the carboxyl terminal existed as an inhibitor of the mitogenic activity of insulin and that removal of the carboxyl domain released the receptor from regulator constraints However, recent suggestions indicate that the extracellular portion of the ß-subunit has been implicated in receptor signalling and internalization Condorelli *et al* (1994) reported the existence of two alternatively spliced human IGF receptor mRNA transcripts which differed by a 3 nucleotide (CAG) start site on the ß-subunit The CAG (-) receptor showed a 2-fold increase in tyrosine auto-phosphorylation, IRS-1 tyrosine phosphorylation and thymidine incorporation relative to the CAG (+) receptor

Lammers *et al* (1989) constructed chimeras in which the cytoplasmic domain of insulin and IGF-I receptors were switched, the ability to stimulate growth was shown to reside in the cytoplasmic domain Maegawa *et al* (1988) also observed an increase in mitogenic activity and no change in metabolic activity when the carboxyl terminal was altered

Interplay of insulin and IGF-I receptor chains has also been found to result in mitogenic activity at physiological concentrations Cross phosphorylation can occur which leads to the phosphorylated IGF-I receptor activating the insulin receptor Heterodimers of the insulin and IGF-I receptor have been reported (Moxham *et al* , 1989, Treadway *et al* , 1989, Schäffer *et*

al , 1993) In this scenario an α-ß heterodimer of an insulin receptor and an α-ß heterodimer of an IGF-I receptor form a hybrid This has been observed for a number of other cell lines in which a heterodimer of IGF/insulin or two distinct IGFs are formed, including NIH3T3 cells, HepG2 cells and Human placental cells (Jonas and Harrison, 1985), IM-9 lymphoid cells (Roth *et al* , 1986) and adult forebrain synaptosomes (Moss and Livingstone, 1993, Shaffer *et al* , 1984) As well as the formation of heterodimers (Ballotı *et al* , 1989), intermolecular transphosphorylation between IR and EGF-Ins hybrid receptor in NIH3T3 has also been reported

In general it would appear that most of the stimulatory activity associated with insulin is mediated *via* the IGF receptor Where insulin acts at physiological concentrations, additional or novel IGF-I receptors are often reported These novel or modified receptors may reflect variations in the requirement of insulin during the fetal stages of growth or specific requirements for certain cell types *e g* in the brain

With the production of recombinant insulin and IGFs, other fields of investigation have yielded interesting information Recombinant IGF-Is can be made with lower cross reactivity with IGF-II receptors (Rosenfeld, 1982) Other investigators have replaced individual ammo acids to produce a recombinant IGF with greater activation/ stability than the natural product

1.6 THE ROLE OF TRANSFERRIN IN SERUM-FREE MEDIUM

Iron is required by eukaryotic and prokaryotic cells where it has several important functions a medium for transport of electrons in the cell and as co-factors for enzymatic reactions Iron has been closely associated with cell growth and DNA synthesis (Anderson *et al* , 1982) In cell culture and especially in SFM the most commonly used chelator of iron is transferrin (Mather and Sato, 1979) Transferrin is also thought to act in the removal of toxic compounds

There is interest as to whether the carrier protein or the iron is the important part Much research now suggests that it is the iron which is required by the cell but that the transferrin protein regulates the availability of iron (Laskey *et al* , 1988) If iron itself is the important component, it should be possible to eliminate the use of transferrin from SFM This is especially desirable since the most widely used source is of human origin, human transferrin is used more often than bovine as it is less expensive and more effective If a chelator is required to regulate bioavailability of iron, use of synthetic compounds would result in a more defined medium, and would be perceived as safer by regulatory authorities

1.6.1 Background

Iron can exist in aqueous solution in two oxidation states Fe^{2+} and Fe^{3+} In solution, hydrolysis and polymerization of aqueous Fe^{3+} to insoluble ferric hydroxides and oxyhydroxides can reduce the potential biological accessibility of iron To avoid this, transferrin has been traditionally used as a means of supplying biologically available iron to cells in culture Transferrins may be divided into several classes depending on the source and the extent of iron bound Within vertebrates there are three classes serum transferrin is the prototype protein in plasma (about 3% of total plasma protein) which mediates transfer of iron to and from various sites in the body, lactoferrin is the iron-binding protein found in milk, granulocytes and other body secretions *e g* tears, ovotransferrin is a major avian protein found in egg white For more detailed information on iron proteins and transport, see Crichton and Charloteaux-Wauters (1987), Thorstensen and Romslo (1990) Iron when conjugated with oxygen can generate hydroxyl radicals, which have a variety of toxic effects on cells (Gutteridge, 1981) Such peroxide formation occurs when synthetic cell culture media are exposed to light The absence of the protective effect of serum or transferrin can make cells grown in SFM very susceptible to peroxide damage

1.6.2 Transferrin Structure

Human serum transferrin is a glycoprotein of 679 amino acids (Mw about 79,500) It is a single poly-peptide and is synthesized primarily in the liver but also in the spleen and bone marrow Transferrin exists in varying sizes and this is thought to be due to varying sialic acid content

The tertiary structure consists of two globular domains (often referred to as N and C termini) each of which contains a binding site for one trivalent atom of iron At both the N and C termini region of transferrin, iron is directly co-ordinated to two tyrosines, one histidine and one aspartic acid and indirectly to an arginine *via* the bicarbonate anion (Thorstensen and Romslo, 1990) The affinity of the ferric iron is so high at physiological pH that there are virtually no free ions in serum Due to the highly anionic state of the oxygen ligands, Fe^{3+} is bound in preference to Fe^{2+} The binding of iron is accompanied by the concomitant binding of a bicarbonate ion for each iron atom bound The binding of the two molecules of iron does not appear to occur to the same degree in ovotransferrin (Butterworth *et al* , 1975) For serum-derived transferrin the C terminal appears to hold a greater affinity for iron The strength of affinities were found to vary depending on the pH

1.6.3 Transferrin Receptor

Transferrin receptors (TRs) are cell surface proteins present in high numbers on healthy dividing cells and their presence is greatly reduced or absent in quiescent non-proliferating cells In structure, the TR is a homodimeric transmembrane glycoprotein covalently bound by one disulphide bond The two subunits have molecular weight of 90 kDa Virtually all cells have TRs and most use the endocytic transferrin cycle for iron uptake (Harford *et al* , 1990)

There are three domains, the C terminal extracellular part (591 amino acid residues), the transmembrane segment consisting of 26 amino acids and the N terminal cytoplasmic domain which has 62 amino acids Like asialoglycoprotein receptors the C terminus is extracellular This part of the subunit is very sensitive to exogenously added trypsin This is unlike many growth factor receptors including EGF and IGF, in which the N terminus is the extracellular component

There are four potential phosphorylation sites but only one serine at position 24 appears to be a target for protein kinase C phosphorylation Expression of transferrin receptor is partially controlled by iron responsive elements Other factors like cytokines may affect transferrin receptor levels Il-1ß and TNF-α decreased the transferrin uptake rate by reducing the number

of receptors on U937 macrophages (Fahmy and Young, 1993) IFN-γ decreased the incorporation by down-regulating the intracellular concentration of ferritin (the major iron storage protein) Effects are specific for different cell types IL-1ß, IL-6 and TNF-α increased the uptake of transferrin into HepG2 cells by increasing the number of receptors (Hirayama *et al* , 1993) Calcium was found to enhance iron uptake by modulating the membrane fluidity (Wright *et al* , 1986)

Transferrin receptors have been studied and two processes describing the release of iron from transferrin-transferrin receptors have been postulated Receptor Mediated Endocytosis (RME) and the Redox Method (Straford-May and Cuatrecasas, 1985, Testa *et al* , 1993)

In the RME scenario, transferrin loaded with iron, binds to the TR at the cell surface The receptor complex then becomes trapped within endocytic vesicles termed endosomes, *via* coated pits and vesicles By means of a proton pumping ATPase of the endosomal membrane, the vesicle becomes rapidly acidified (pH 5 - 5 5) At this pH, the iron has little affinity for transferrin and is mobilized from transferrin and transported across the endosomal membrane into the cytosol By unknown means the apotransferrin - TR (apoTf-TR) complex is sorted into endocytic vesicles and escapes lysosomal degradation The endocytic vesicles interface with the plasma membrane and the apoTf-TR complex is exposed to the extracellular pH At this pH, apoTf has lost its affinity for the TR and dissociates This has been suggested for K562 cells (Klausner *et al* , 1983)

There are several problems with this proposed mechanism for some cell lines (Morley and Bezkorovainy, 1985) The uptake of iron takes only a matter of minutes (3 - 4) at 37°C The time taken for the pH in the vesicles to be reduced would take considerably longer Also lowering of the extracellular pH is insufficient to cause release of the iron (Thorstensen and Romslo, 1990)

The Redox method centres around the oxidative potential of the iron molecule itself The process of endocytosis is the same as for RME Before the transferrin-transferrin receptor (Tf-TR) becomes trapped with endocytic vesicles, separation of the iron from the Tf-TR complex is proposed to be due to the concerted action of protons and reducing equivalents which are furnished by a nearby NADH ferricyanide oxidoreductase redox system The change in redox potential causes a destabilization of the iron-Tf bond and results in the reduction of the iron

molecule The iron may then be bound to a membrane carrier specific for Fe^{2+} The iron would then be translocated across the membrane to the cytosol where it is picked up by an iron acceptor

How the iron signal is directed into the cell remains unclear (Crichton and Charloteaux-Wauters, 1987, Klausner *et al* , 1993) When iron is released from the Tf - Tf-receptor complex, there are three target pools The iron may be utilized as a component or co-factor of cellular proteins It may be stored, primarily by ferritin (also in haemosiderin) This may occur by the assembly of apoferritin subunits surrounding preformed micellar iron complexes or by the entry of Fe^{2+} into a protein shell of the apoferritin where it is oxidized, hydrolysed and finally polymerized into a ferric oxyhydroxy polymer (Crichton and Charloteaux-Wauters, 1987) The resulting polymer would be too big to escape from the ferritin complex Iron may be used in the regulatory pool where there is interplay between iron responsive element-binding proteins (IRE-BPs) and iron responsive elements to control the levels of iron in the cell When there is plenty of cellular iron, IRE-BPs have a high affinity for aconitase activity and little affinity for RNA binding When iron becomes depleted, the IRE-BPs have little affinity for aconitase and high affinity for binding RNA By binding to the mRNA of ferritin, a positive translation promoting site is blocked, thereby reducing the biosynthesis of ferritin IRE-BPs also bind to a 5' untranslated region of the transferrin receptor mRNA, blocking the binding of instability determinants thereby stabilizing the RNA transcript This results in increased receptor transcription (Hirling *et al* , 1994)

Cellular uptake of iron *via* transferrin does not account for all the iron uptake processes Other mechanisms which are TR-independent and transferrin-mdependent have been reported for a wide variety of cells including cultured fibroblasts (Oshiro *et al* , 1993), HL60 lymphoblastic cells (Chitambar and Zivkovic, 1987), K562 Cells (Inman and Wessling-Resnick, 1993), human hybridoma cells (Ill *et al* , 1988), rat hepatocytes (Thornstensen and Romslo, 1988), L1210 leukemia cells (Basset *et al* , 1986), human melanoma cells (Richardson and Baker, 1992), CHO cells (Chan *et al* , 1992) and reticulocytes (Hodgson *et al* , 1994) Bradbury *et al* (1994) found that transferrin-dependent and transferrin-mdependent mechanisms existed in a variety of cell types of which the greatest capacity for non transferrin-bound iron were cells from liver and renal cortex A combination of uptake mechanisms may be used by cells such as hepatocytes (Thorstensen and Romslo, 1990) Some cell lines produce their own transferrin *e g* Reuber Liver cells (Shapiro and Wagner, 1989), mammary epithelial cells (Lee *et al* ,

1987), $CD4^+$ lymphocytes (Lum *et al* , 1986), HeLa (Kriegerbeckova *et al* , 1993), rat hepatocytes (Mitaka *et al* , 1993) and Rhodamine fibrosarcoma cells (Nagao and Nishikawa, 1989) Chitambar *et al* (1987, 1989, 1990) found that HL60 cells contained soluble transferrin receptors in the conditioned medium These soluble transferrin receptors occur by proteolytic digestion at the extracellular plasma membrane and may have a role in scavenging iron from the environment This has also been observed for erythroid progenitor cells during differentiation (Shintani *et al* , 1994)

Conrad *et al* (1994) suggested a transferrin-independent iron transport system which was mediated by a sequential passage of iron to integrin, mobilferrin and ferritin in a human-derived erythroleukemia cell line

Amouric *et al* (1984) found that two iron solutions of ferrous sulphate and ferric chloride could replace transferrin in supporting the growth of a human colon adenocarcinoma cell line (HT 29) The ability of simple ferric compounds to replace transferrin has been reported for a variety of other cells (Brock and Stevenson, 1987 and Laskey *et al* , 1988) Insoluble iron has even been suggested to stimulate growth in a mouse hybridoma, PLV-01 (Kovar, 1990)

Where simple iron salts were insufficient to replace transferrin, more complex synthetic chelators have been used Tsao *et al* (1987) found that while, several inorganic iron salts (ferric ammonium citrate and ferrous sulfate) could not support the growth of non-tumorigenic anchorage-dependent hepatic epithelial cells in SFM, ferric pyridoxal isonicotinoyl hydrazone (Fe-PIH), a lipophilic iron complexing agent, could be used but at a 1000-fold increase in concentration

Laskey *et al* (1988) looked at the effect of an iron chelator to stimulate the growth of Friend murine erythroleukemia (mel) cells, Raji cells (B lymphoblast from Burkitts lymphoma) and human peripheral blood lymphocytes The chelator, ferric salicylaldehyde isomcotinoyl hydrazone (Fe-SIH) was shown to promote growth independently of the TRs While Fe-PIH could replace transferrin for embryonic metaphrenic mouse cells (Landschulz *et al* , 1984), it could not be substituted for transferrin on embryogenic mesoderm cells on a hydrated collagen matrix (Sanders and Cheung, 1988)

In trying to replace human transferrin, Ill *et al* (1988) found that while human x human and human x mouse hybridomas could grow in SFM with iron complexes (ferric citrate, iron choline citrate and Fe-PIH), no growth was observed with mouse x mouse hybridomas Replacement of transferrin did not affect monoclonal antibody production However, others have been able to grow mouse hybridomas without transferrin Yabe *et al* (1987) looked at the effect of low molecular weight chelators such as Fe(III)-glycylglycine, Fe(III)-immodiacetic acid and Fe(III)-dihydroxethylglycine When growing mouse hybridomas, these chelators could replace transferrin Darfler (1990) used sodium mtroprusside instead for the growth of murine hybridomas without affecting monoclonal antibody production

Kovar and Franek (1987), used ferric citrate and found that over 30 passages, growth and monoclonal antibody production was maintained, indicating that the ability to support growth was not due to residual transferrin Fe_2SO_4 was also used to replace transferrin for the growth of mouse hybridomas (Shinmoto *et al* , 1988)

Metcalfe *et al* (1994) found that 4 alternatives to transferrin including ammonium ferrous citrate could support growth in a static culture but only 2-hydroxy-2,4,6-cyclohepatrin-1-one (Tropolone) could replace transferrin in large-scale agitated cultures of mouse myeloma cells This could imply that precipitation of the iron molecule is important for the uptake of iron So although simple iron complexes may replace transferrin in static cultures, more complex iron carriers may be needed for suspension cultures

For many cell lines, the ability to use simple ferric salts or more complex synthetic chelators as an iron source, regardless of the method of uptake has removed the requirement for transferrin In some cases where the role of transferrin cannot be replaced by other chemical chelators, bovine-derived as opposed to human transferrin, or the use of lactoferrin (bovine or recombinant) may be an alternative (Perez-Infante and Mather, 1982, Hashizume *et al* ,1983, Ill *et al* , 1988 and Yamada *et al* , 1990)

1.7 THE ROLE OF BOVINE SERUM ALBUMIN IN SERUM-FREE MEDIUM

Serum albumin is the most abundant protein in blood plasma. *In vivo*, albumin has many functions. It provides approximately 80% of the osmotic pressure in blood and contributes to cellular nutrition. It regulates free calcium concentrations and transport of fatty acids, tryptophan, toxic compounds, steroid hormones and metal ions and acts as a source of amino acids following proteolysis.

Albumin has many uses. Thousands of kilograms are produced a year for therapeutic purposes (for example to improve circulatory performance, a blood extender). It is used as a growth support for mammalian cells and microorganisms, as a carrier/stabilizer of growth factors and hormones and as a blocker in immunological studies (as a non specific binder in radioimmunoassays and separation processes). BSA has been incorporated into SFM for a variety of cell lines including human muscle satellite cells (Ham *et al.*, 1988), mouse vaginal epithelial cells (Iguchi *et al.*, 1987) and haempoietic cells (Guilbert and Iscove, 1976) and some hybridomas (Jäger *et al.*, 1988).

1.7.1 Background

BSA was originally isolated on a large-scale by Cohn *et al.* (1946) using low temperature ethanol precipitation. Other methods of isolation have since been developed (heat-shock treatment and salt fractionation). Due to the 'stickiness' of albumin, a completely pure preparation is difficult to obtain, generally between 96 to 99% albumin. The remaining contaminants of the albumin are naturally bound ligands such as bilirubin, fatty acids, hormones, globulins and metal ions. Chromatography is becoming a viable alternative to the more traditional methods. Stoltz *et al.* (1989) used chromatographic techniques to obtain human albumin from plasma with 99 - 100% purity.

1.7.2 Structure

Albumin is a non-glycosylated single chain protein of about 585 amino acids residues. Serum albumins belong to a multigene family of proteins that include α-protein and vitamin D binding protein. Its chief traits are those of an acidic, very soluble, stable protein. At low pH (1-2), the albumin molecule can reversibly denature and renature. At high pH (9-11), it is relatively unharmed but can undergo deamination of disulphide bonds. It is one of the few secreted proteins which lack carbohydrate. It contains 17 disulphide bonds which influence the structure

and stability of the protein. The 17 disulphide bonds determine the formation of a series of nine loops, which are broken up as three domains of three loops. The three domains are semiautonomous but homologous. They are capable of folding to form hydrophobic pockets. In addition to stability, the total high charge (about 185 ions per molecule at pH 7) make it very soluble. There is evidence to suggest the existence of albumin specific cell surface receptors. Schnitzer *et al.* (1988) found a single 60kDa cell surface glycoprotein which was precipitated in common with three agglutinins that competed with albumin for uptake in monolayers of microlayers of microvascular endothelial cells. This was later called Albondin. Other putative albumin-binding proteins (SPARC (secreted protein acidic and rich in cysteine), gp60, gp30 and gp18) have been found. It was suggested that albondin mediates native albumin binding which enhances its transcytosis and capillary permeability, while gp30 and gp18 mediated the binding, endocytosis and degradation of modified albumins (Schnitzer and Oh, 1994). These receptors would be necessary as part of the cellular function of endothelial cells in the transport from capillary vessels to interstitial fluids. Albondin and SPARC appears to be selectively expressed in tissues with microvascular beds lined with a continuous endothelium which bind and transcytose albumin. The albumin-binding proteins gp30 and gp18 have been found on fibroblasts and macrophages which may indicate a scavenging role for these cells (Sage *et al.*, 1984; Goldblum *et al.*, 1994; Schnitzer and Bravo, 1993; Schnitzer *et al.*, 1992).

Species differences exist in the structure of albumin and as a result the extent of ligand binding varies. Alignment of human, bovine and rat albumin shows about 80% homology between human and bovine albumins, 80% between human and rat, and 63% homology conserved in all three. For a more detailed information on albumin, its structure and ligand binding properties see reviews by Peters (1985), Kragh-Hansen (1981) and Brown and Shockley (1977).

1.7.3 Ligand Binding

The most unique feature of albumin is its ability to bind a wide variety of biological materials, generally it has highest affinity for small negatively-charged hydrophobic molecules. The flexibility of the molecule means it can bind a wide variety of compounds. The presence of hydrophobic pockets makes it an ideal carrier of compounds which have limited solubility in aqueous solutions. Interactions may have low or high specificity. Despite the similarities of amino-acid sequences and the retention of the characteristic repeating series of disulphide bonds in mammalian albumins, there may be marked differences in ligand binding *e.g.* palmitate binds

more tightly to bovine than to human or murine albumins and *vice versa* for oleate (Richieri *et al* , 1993), bilirubin binds more strongly and in a different rotation to human than to bovine albumin Hematin is tightly bound only to albumins derived from primates (Adams and Berman, 1980)

1.7.3.1 Fatty acid binding

Long-chain fatty acids are quantitatively the most important component bound to albumin Albumin by binding to fatty acids can control the amount of free fatty acids For cells growing in culture, fatty acids become toxic if the concentration is too high At a cellular level, fatty acids affect the oxidative phosphorylation processes in the mitochondria Albumin thus acts as a pool of fatty acids When the free fatty acids are used up, thermodynamics cause some of the bound fatty acids to be released and hence available to the cells

The normal loading is about 2 fatty acid molecules per molecule albumin Serum albumin can bind up to 6 fatty acids strongly and about 20 weakly The extent of fatty acid binding depends on the aliphatic chain length The longer the fatty acid chain, the stronger the binding affinity Fatty acids such as oleic, stearic, palmitic and linoleic acid bind relatively tightly, while decanoic and octanoic bind at lower affinities

De Miranda *et al* (1976) suggested that a separate binding site existed for fatty acids containing 4 or less carbon atoms The primary binding sites on bovine albumin are located at the C terminal loops, 7 and 8 Attachment of one or two long-chain fatty acids is known to be important in maintaining the native structure of albumin Their binding causes the protein to become more spherical and thus less susceptible to proteolysis Esterified fatty acids also bind to albumin but with lower affinities

1.7.3.2 Other ligands

Albumin has two binding sites for bilirubin These are non-competitive with fatty acids unless there is a high concentration of the latter Bilirubin binding occurs near loop 4 of bovine albumin

Steroid hormones bind to albumin with a low affinity These include estradiol, aldosterone, testosterone and progesterone There is no specific site for steroid binding Progesterone and deoxycorticosterone bind to the same site while testosterone binds to a different site Steroid hormones may compete with fatty acids and bilirubin for binding

L-Tryptophan and L-thyroxine are the only amino acids which bind to albumin They share the same binding site with each other and compete with short-chain fatty acids and numerous organic anions for these sites Thus the amount of ammo acids bound to albumin will vary depending on the amount of other ligands present Other analogues of tryptophan and thyroxine also bind weakly These include catecholamines, folate and vitamin B-12

The N-terminal provides binding sites for a variety of metal ions including Ca^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} About half the plasma calcium is bound to albumin but the binding is not strong or specific Only 10% of plasma copper binds to albumin

Albumin also binds a variety of drugs (penicillin, barbiturates, salicylate, warfarin and digitoxin) and polyaromatic dyes such as fluorescein and bromocresol green Most are bound at low specificity sites, probably in the region of hydrophobic domains

1.7.4 Biological activity associated with albumin

With the ligand binding capabilities of serum albumin, it is no wonder that it is so commonly used in cell culture and especially in serum-free medium However, the exact role of albumin in such systems is ambiguous Mitogemc activity has been associated with albumin, but is this due to the albumin molecule itself or as a result of the ligands it carries? To add to the confusion, the effects appear to vary depending on cell type and the purity of the albumin Reports appear regularly in the literature comparing different batches of albumin Not only is the growth affected by the different methods of purification, but also by batch-to-batch variations from company to company

The fact that BSA is used in milligram quantities in culture as opposed to microgram quantities for growth factors, has led some researchers to believe that the growth stimulating ability of albumin is due to some as yet unidentified contaminating factor

Nilausen (1978) investigated the role of fatty acids (lysolecithin in particular) in the growth promoting effects of serum albumin on hamster cells Dialysed serum albumin had growth stimulating activity but this was lost when the fatty acids were removed When the isolated and purified fatty acids were recombined with the fatty acid free albumin, the growth promoting activity was restored All unsaturated fatty acids were stimulatory whereas, all saturated fatty acids were growth inhibitory except stearic acid which was stimulatory at low concentrations

When comparing dialysed to untreated albumin, the dialysed albumin was found to have double the growth promoting activity of the untreated albumin for nearly all concentrations tested This was presumably due to the removal of some inhibitory factor Indeed, Hanson and Ballard (1968) suggested that dialysis removed citrate, lactate, pyruvate and some ferric ions which may have been inhibitory

Ham (1963) used linoleic acid corn oil to replace albumin for the growth of a chinese hamster strain (CHD-3) Oleic acid and esters of hnoleic acid and linolenic acid could not replace BSA For HeLa cells, arachidonic acid and linoleic acid coupled to albumin were found to stimulate growth while the albumin alone was not (Gershenson *et al* ,1967) Jager *et al* (1988) found BSA-oleic acid complex was important for the long-term (more than 10 passages) growth of Ag8-myeloma cells in SFM BSA without oleic acid had no affect on the growth of these cells

Similar results supporting the role of lipids in the action attributed to albumin were obtained with Yoshida sarcoma cells (Yamane *et al* , 1975), SV40 transformed cells (Rockwell *et al* , 1980) and human diploid fibroblasts (Kan and Yamane, 1982) Supporting the idea of ligands rather than the albumin being responsible for the biological activity associated with BSA, batch-to-batch variability as been reported (McKiernan and Bavister, 1992, Tomooka *et al* , 1985)

1.7.5 Replacement of BSA

Where fatty acids have been found to be responsible for the activity associated with albumin, a variety of lipid binding molecules have been utilized to replace albumin ß-lactoglobulin which possesses the ability to bind and release fatty acids like albumin, could be used to replace fatty acid free albumin with the same growth stimulation Anderson *et al* (1990) reported that casein could replace BSA in SFM to cause contraction of human foreskin fibroblasts and rabbit aortic smooth muscle cells

Cyclodextrins have also been used to replace albumin Oleic acid and hnoleic acid were complexed to ß-cyclodextrm and used to promote the growth of human lymphoblast cells (100μg/ml) and human diploid fibroblasts (Szejtli, 1986) In addition α-cyclodextrin was used to prolong growth and monoclonal antibody production (Minamoto and Matsugi, 1984)

In some cases however, the presence of fatty acids was either not responsible for the activity associated with BSA or was inhibitory Norkin *et al* (1965) reported that growth of macrophages was enhanced slightly when fatty acids were removed from albumin without denaturing The improvement in growth was not necessarily due to the albumin molecule itself being stimulatory The basal medium contained 30% chicken serum from which the albumin could have been binding some inhibitory component in place of the fatty acids

Chiang *et al* (1993), observed a higher inward Ca^{2+} current in chicken granulosa cells when using BSA fatty acid free (BSA-faf) than BSA Addition of oleic acid decreased the potency of BSA-faf

Melsert *et al* (1989) found albumin from rat testicular fluid to enhance leutinizing hormone-stimulated pregnenolone production by immature Leydig cells *in vitro* The stimulatory activity was not due to fatty acid or the source of albumin as albumin from rat, bovine, and human sera were as good, suggesting that the activity was due to the albumin itself

Polet and Spieker-Polet (1975) and Spieker-Polet and Polet (1976) found that serum albumin was essential for the growth of activated human lymphocytes Comparing rabbit, human and bovine serum albumin revealed no difference in growth stimulating ability BSA fraction V and BSA-faf stimulated DNA synthesis suggesting that the fatty acids were not the basis of the growth-stimulating effect Similarly incorporation of growth factors and hormones had no effect on the ability of albumin to stimulate growth This led to the conclusion that either the albumin molecule itself was responsible (directly or by permissive means) for growth or that some other stimulatory factor was tightly bound to albumin

Further investigations into the role of albumin revealed that albumin from several sources (human, bovine and rabbit) could stimulate growth equally well at low concentrations At higher levels, the results varied This was suspected to be due to artifacts By means of molecular sieving, ion exchange chromatography, isoelectric focusing, charcoal treatment, acetone precipitation and reduction, no other factor was found to be responsible for growth stimulation

In addition to the presence of fatty acids, investigators have found a variety of other ligands to be responsible for the activity of albumin Congote (1984) extracted erythrotropin from fetal bovine serum and found it could stimulate fetal calf liver cells In a subsequent experiment, Congote (1987) was able to isolate an erythrotropin-like factor from BSA fraction V Although

all preparations stimulated thymidine uptake in erythroid cells from fetal bovine liver, cruder preparations showed greater stimulation Purification of albumin using reverse phase HPLC revealed a factor that was very similar to bovine serum erythrotropin Congote concluded that the growth stimulating effect of many albumins may be due to the presence of erythrotropin-like factors

Thomassen (1989) investigated the variable responses of Rat Tracheal Epithelial cells (RTE) to BSA in serum-free culture He compared crude fraction V, essentially globulin-free, fatty acid free, and essentially globulin and fatty acid free BSA Growth response was found to vary depending on the purity of the albumin, on the concentration and on the presence or absence of cholera toxin

Barlian and Bols (1991) investigated the growth promoting effect of BSAs which could support salmomd cells in serum-free culture They found differences existed in the growth stimulatory activity depending not only the purity of the albumins but also in the method of preparation Albumins initially purified by cold alcohol precipitation were found to be more stimulatory than those prepared by heat shock treatment

Kane (1990) investigated the growth control in pre-implantation embryos Blastocyte growth in medium tended to be variable and this was pinned down to variations in the batches of BSA used Further investigations revealed that the contaminating factor was citrate Whether the total activity of albumin in this system is due to citrate is unknown Citrate may help to transport ions or stimulate fatty acid synthesis

Tigyi and Miledi (1992) found lysophosphatides bound to BSA to be responsible for activating membrane currents in *Xenopus* Oocytes and to cause neurite retraction in PC12 Pheochromocytoma cells Extraction of lipids with methanol removed activity When synthetic lysophosphatides were added to inactive BSA-faf, activity was restored

The evidence to date would support the fact that it is the impurities bound to albumin which have growth stimulating effects on cells in culture However for some cell lines it may be the molecule itself which is stimulatory as suggested by Polet and Spieker-Polet (1975) How the albumin molecule itself could exert a stimulatory effect is, as yet, not known

1.8 INTRODUCTION TO THE RESEARCH WORK CARRIED OUT IN THIS THESIS

In this thesis, alternatives to fetal bovine/calf serum in mammalian cell culture were investigated As outlined in section 1 1, there are many disadvantages to the use of serum in supporting growth and metabolic activities of cells in culture However, the design of a SFM for a particular cell line may in itself be problematic

The aims of this thesis were as follows

1 To develop a SFM for normal rat kidney (NRK) cells A SFM had been designed for a subclone of the cell line, NRK - 49F cells (Rizzino, 1984) However, when tested, the parental cells did not grow in this SFM As the NRK cells would not even remain viable in the basal medium alone, an alternative approach was devised Cells were grown in the presence of small amounts of serum, to detect factors which were important for cell growth by adding growth stimulatory factors The level of serum could be reduced while still maintaining growth and viability The eventual aim was to delete the serum totally from the medium, which would result in the formulation of a SFM that would support the growth of the parental NRK cells When a SFM was obtained, it would be necessary to ensure that the growth promoting effects were not due to serum contaminants residing in the cells This would be done by subculturing the cells through several passages

2 As a result of experiments carried out in the earlier stages of the thesis on NRK cells in a low serum-supplemented medium, BSA was found to be stimulatory It was hoped to see if the activity associated with BSA could be isolated from the BSA molecule As mentioned in section 1 7, BSA is a very sticky molecule and its apparent activity has most commonly been due to the factors that it carries rather than the albumin molecule itself *e g* fatty acids, phospholipids, citrate and factors produced during the clotting process Methods already used to isolate the activity from albumin that would be attempted, included the following lipid extraction, reverse-phase HPLC, gel filtration In addition, affinity chromatography would also be used

If it were possible to separate the activity from the BSA molecule, it was hoped to replace the active component with a synthetic analogue and incorporate it into a SFM for NRK cells If the BSA molecule was found to be necessary for biological activity of the active factor, then it may be possible to replace albumin with other carrier molecules, for example cyclodextrins

For some existing SFM, it was hoped to replace the two most universally used components in SFM, insulin and transferrin to produce a defined medium devoid of animal-derived proteins The two cell lines chosen were Chinese Hamster Ovary (CHOK1) and Madm-Darby Canine Kidney (MDCK) cells

3 CHOK1 cells are industrially important due to their growth characteristics, safety, ease of transfection and high productivity of recombinant gene products Several SFM existed for CHO cells but all required insulin and/or transferrin The SFM designed by Mendiaz *et al* (1986) was chosen as it was based on CHOK1 cells and because insulin and transferrin were the only ammal-derived proteins present in the medium The SFM contained bovine-derived insulin and transferrin and growth was found to be dependent on insuhn If the bovine-derived insulin could be replaced with a recombinant IGF or recombinant msulin, then a SFM devoid of animal-derived proteins would be formed as CHOK1 cells had been shown not to depend on transferrin when ferrous sulphate was present in the medium

4 MDCK cells are industrially important in the production of canine vaccines MDCK cells have been grown in a SFM designed by Taub *et al* (1979) The cells were found to be very dependent on the presence of human transferrin with little dependence on insulin The removal of msuhn and the replacement of transferrin would upgrade the SFM to being devoid of animal-derived proteins It was intended to try to replace transferrin with simple inorganic and organic iron salts and compounds If these were not found to be successful, more complex synthetic chelators would be attempted If a replacement were found it would be necessary to prove the continued ability of the compound to support growth in the absence of transferrin by subculturing the cells for several passages with the transferrin replacement

2.0 MATERIALS AND METHODS

2.1 WATER

For all reagents and media, water was purified by a reverse osmosis system (Millipore, Elgastat UHP) to produce ultrapure water (12 - 18 mΩ-cm resistance)

2.2 GLASSWARE

All glassware which was to come in contact with cells was treated in the following way The bottles and lids were soaked in a 0 2% warm solution of RBS (AGB Scientific) for 60 to 90 minutes This is a deproteinizing agent which removes proteinous material from the bottles Following scrubbing and several rinses in tap water, the bottles were cleaned in a washing machine The first cycle used NEODISHER, an organic, phosphate-based acid detergent A further cycle of two rmses in distilled and one in ultrapure water left the bottles ready for use Bottles were sterilized by autoclaving for 15 minutes under pressure of 1bar at 121°C

Bottles used for the storage of media for serum-free work were purchased specially for the purpose These bottles were not allowed to come in contact with serum containing media The bottles were soaked and washed as above except that there were four rinsing stages after which the bottles and lids were dried out The bottles and lids were then rinsed three times in ultrapure water and sterilized by autoclavmg

2.3 MEDIA PREPARATION

Media for general cell culture purposes were made up in 5L volumes according to standard laboratory practices (see Table 2 3) The pH was adjusted by the addition of sterile 1 5N NaOH or 1 5N HCl and brought up to a final volume of 5 litres Although all components and additives were sterile, the 1X media was filtered through a 0 22μm filter and stored in 500ml bottles at 4°C Sterility checks carried out included, turbidity, pH change, Columbia blood agar plates, Sabauraud dextrose and Thioglycollate broths

The expiry date on all 10X media was checked and at least a 2-months leaway was given so that media components should not have broken down and resulted in a loss of activity For the different cell lines, variations in the buffering of the media were cited when making up a SFM To allow for this, 500ml of 10X media was made up to 4 5 litres *i e* a 1 1X stock When the appropriate concentrations of buffering agents were added depending on the cell line, the pH of the medium was adjusted The basal medium was brought to a 1x stock by addition of sterile ultrapure H_2O

Media was stored at 4°C. The media was also stored in the dark to prevent the possible formation of free radicals which could be detrimental to cells under serum-free conditions or to prevent the degradation of light sensitive vitamins (B and C group).

Table 2.3 Media preparation

10X MEDIA	MEM (500ml)	DMEM (500ml)	Ham's F12
Catalogue no.	042-01430M	042-02501M	074-01700N
UP H_2O	4200ml	4300ml	4700ml
1M HEPES	100ml	100ml	100ml
7.5% $NaHCO_3$	45ml	45ml	45ml
MEM NEAA	50ml	--	--

Abbreviations: MEM = Minimal Essential Medium; DMEM = Dulbeccos' modified essential medium
MEM NEAA = MEM Non Essential Amino Acids (Gibco: Cat. No. 043-01140). All 10X media were supplied by Gibco Life Sciences. Hams' F12 was supplied in powder form.

2.4 REAGENTS AND THEIR RECONSTITUTION

The following tables provides information on stock reagents (source and catalogue numbers) and reconstitution. All stock solutions (except sera) were filter sterilized and stored at 4°C or -20°C.

Table 2.4.1 General cell culture reagents

PRODUCT	SIZE	CAT. NO.	SUPPLIER
L-Glutamine	100ml	043-05030H	Gibco
Sodium Pyruvate	100ml	043-01360H	Gibco
Trypsin	100ml	043-05090H	Gibco
Trypsin Inhibitor	100mg	T6522	Sigma
DMSO	100ml	D2650	Sigma
HEPES	1Kg	H9136	Sigma
$NaHCO_3$	1Kg	30151	BDH
HCl	2.5L	37021	RDH
NaOH	1Kg	30620	RDH
Donor Horse Serum	500ml	29-211-54 (lot 9120952)	Flow Laboratories
Fetal Calf Serum	500ml	Batch 41925	Northumbria Biologicals
PBS A	100 Tablets	BR14a	Oxide
EDTA	100ml	ED6758	Sigma

Table 2.4.2 Reagents used in albumin studies

PRODUCT	SIZE	CAT NO	SUPPLIER
HSA fraction V	5g	A1653 (lot 86F-9383)	Sigma
HSA fatty acid free	5g	A3782 (lot 119F-9303)	Sigma
Bovine Albumin Fraction V	5g	A4919 (lot 110H-04635)	Sigma
BSA fatty acid free	5g	82-042-2 (lot 303)	Pentex
BSA fraction V, initial isolation by Cold alcohol precipitate	5g	A4503 (lot 12H0283)	Sigma
BSA faf derived from A4503	5g	A6003 (lot 119F306)	Sigma
HSA fraction V	5g	A1653 (lot 106F9333)	Sigma
HSA faf derived from A1653	5g	A1887 (lot 42H9313)	Sigma
ESA fraction V	5g	A9888 (lot 37F9326)	Sigma
ESA faf derived from A9888	5g	A5280 (lot 47F9303)	Sigma
BSA fraction V, initial isolation by heat shock	10ml of 35% solution	A7409 (lot 102H9401)	Sigma
BSA fraction V, initial isolation by salt fractionation	5g	A3675 (lot 110H0783)	Sigma
Oleic acid	100mg	O4379 (lot 69F-84615)	Sigma
Phosphatidylserine	100mg	P5660 (lot 69F-8371)	Sigma
Phosphatidylcholine	100mg	P0763 (lot 109F-8365)	Sigma
Cholesterol	5g	C7402 (lot 58F-72555)	Sigma
Pentex Ex-cyte (III)	5ml	82-019 (lot 210)	Pentex
Endotoxin Standard	2μg at 6000EU/mg	210-SE (lot 105F-6828)	Sigma
L-α-phosphatidic acid, Dioleoyl	10mg	P2767 (lot 61H8362)	Sigma
L-α-lysophoshatidic acid, Oleoyl	5mg	L7260 (lot 91H8443)	Sigma
Caprylate	10g	C3901 (lot 18F8350)	Sigma

All solutions were dissolved without difficulty in sterile PBS A or basal medium (ATCC) except for the following Lysophosphatidic acid was dissolved in a mixture of chloroform, acetone and methanol at a ratio of 95 5 5 Phosphatidic acid was dissolved in chloroform initially and diluted down in basal medium Oleic acid, cholesterol, phosphatidylserine and phosphatidylcholine were reconstituted as described in section 2 11 Additional basal media used in experiments were as follows **BME** (Gibco, 041-02300M), L-15 (Gibco, 041-01415M), RPMI-2650 (Gibco, 042-02511H), GMEM (Gibco, 041-01710M), McCoys 5a (Sigma, M8403), Earls balanced salts (Gibco, 24010-043)

Table 2.4.3 Reagents used in insulin studies

PRODUCT	SIZE	CAT NO	RECONSTITUTION
Bovine Insulin	100mg	I1882 (lot112H9402)	reconstitute in 100µl glacial acetic acid and dilute down in PBS A initially to 5mg/ml
Hybrimax bovine Insulin	100mg	I4011 (lot 79F00925)	
rec human IGF-I	10µg	1048058 (lot14706100)	Reconstitute in 5µl 10mM HCl Dilute down in PBS A
rec human IGF-II	10µg	I2139 (lot 62H00501)	Reconstitute in 1ml of 1mg/ml BSA faf and 3µl 1 5M HCl Dilute down in basal medium
human rec Insulin	100µg 50mg	I0259 (lot 44F01563) I0259 (lot 53H0057)	Reconstitute in 10nM HCl and dilute down in PBS A to 500µg/ml
Ovine Insulin	5mg	I9254 (lot 57F0667)	Reconstitute in 7µl of 1 5M NaOH and dilute down with PBS A to 500µg/ml
Equine Insulin	5mg	I9129 (lot 80H0492)	Reconstitute in 7µl 1 5M HCl and dilute down in PBS A to 500µg/ml
Porcine Insulin	5mg	I3505 (lot 50H0774)	Reconstitute into 6µl of 0 01M HCl and dilute down in PBS A

Except for recombinant human IGF-I, all other insulins and IGF-II were supplied by Sigma IGF-I was supplied by Boehringer Mannheim

Table 2.4.4 Reagents used in transferrin studies

PRODUCT	SIZE	CAT NO	SUPPLIER
apo - Transferrin	5g	T8027	Sigma
partially-saturated Transferrin	5g	82-0425-02	Pentex
fully-saturated Transferrin	5g	T1283 (lot 30H00155)	Sigma
Sodium nitroprusside	25g	S0501 (lot 121H2608)	Sigma
Iron choline citrate	100g	I7756 (lot 84F06371)	Sigma
Ferric citrate	100g	F2877 (lot 82H0695)	Sigma
Ferric nitrate	100g	F8508 (lot 31H05066)	Sigma
Ferric ammonium sulphate	250g	F8884 (lot 119F06215)	Sigma
Ferric ammonium citrate	100g	F5879 (lot 82H0217)	Sigma
Ferrous sulphate	250g	F8633 (lot 50H08335)	Sigma

All transferrins were dissolved into ATCC and stored at 5mg/ml stock solutions All iron compounds were dissolved in ultrapure H_2O at 5mg/ml Dilution to 1mg/ml was made in ultrapure H_2O and all subsequent dilutions were made in basal medium

Table 2.4.5 Reagents required for serum-free (SF) and low serum (LS) assays

PRODUCT	SIZE	CAT NO	RECONSTITUTION
Dexamethazone	1mg	D8893	Add 1ml absolute ethanol, dissolve, add 49ml sterile medium Stock = 20μg/ml
ß-Estradiol	1mg	E2257	
Hydrocortisone	1mg	H0135	Dissolve in 1ml absolute ethanol Add 19ml sterile medium Stock = 50μg/ml
Prostaglandin - E1	1mg	P7527	
Insulin	0 1g	I1882 I4011	Add 9 9ml of sterile H_2O followed by 100μl of glacial acetic acid Stock = 10mg/ml
Sodium Selenite	1mg	S5261	Add 20ml sterile medium Stock = 20μg/ml
Bovine Serum Albumin	5g	A4919 82-0425-02*	Dissolve in 25ml of sterile PBS A or medium Stock = 200mg/ml
Bovine Transferrin	10mg	T5391 82-0554*	Dissolve in 50ml sterile medium Stock = 200μg/ml
Triido-L-thyronine	0 1g	T6397	Add 1ml 1N NaOH, gently rotate to dissolve, add 49ml sterile medium Stock = 2mg/ml
Ex-Cyte-V*	1g	81-125-1	Dissolve Ex-cyte V in 10ml sterile medium Dilute Ex-cyte III 1ml in 10ml sterile medium Stock = 1mg/ml
Ex-cyte-III*	1g	82-019	
EGF	0 1mg	E4127	Dissolve in 20ml sterile medium Stock = 5μg/ml
α-FGF ß-FGF	1μg 1μg	F5267 F5392	Dissolve in 10ml sterile medium with 1mg/ml BSA
Heparin	25,000U	H8514	Dilute to working stock in basal medium
Interleukin-1α (LS assays) Interleukin-1α (SF assays)	1000U 1μg	1059408 BDP14 (lot AC220 29)	Dissolve in 1ml HEPES buffered PBS A and 1mg/ml BSA, dilute down in basal medium
Interleukin - 1ß	1000U	1059394	
IGF - I	10μg	IP9010	Dissolve in 1ml 10mM HCl and 1mg/ml BSA Dilute down in basal medium
bovine HDL	10μg/ml	L2014	Dilute down in sterile medium
Laminin	1mg	L2020	Dilute to 100μg/ml in 10ml sterile PBS A and store at -70°C Thaw out gradually Use minimal volume to coat dish (*e g* 250μl per 1 76cm² plate (24-well)) Allow to air dry
Fibronectin	5mg	F4759	As for Laminin but store at -20°C
PDGF	100U 1μg	G516a[1] G518a (lot 010201)	Dissolve into PBS A with 1mg/ml BSA fatty acid free Dilute down in basal medium

* Pentex products supplied by Bayer 1 supplied by Promega, IL-1α and IL-1ß supplied by Boehringer Mannheim, All other factors supplied by Sigma Attachment factors were used in conjunction with serum-free and in some serum reduced experiments The concentration of attachment factor was usually related to the area of the surface to be coated and not to volume Attachment factors were always used as a pretreatment and not in the actual growth medium (except where laminin was added directly to the growth medium) All attachment factors were supplied by Sigma

Table 2.4.5 continued. Reagents required for serum-free and low serum assays

PRODUCT	SIZE	CAT. NO.	SUPPLIER
BSA - linoleic acid	5g	L8384	Sigma
BME vitamins	100ml	043-01040H	Gibco
MEM vitamins	100ml	043-01120H	Gibco
Interleukin - 4 (human recombinant)	500U/ml	1385984 (lot 14757800)	Boehringer Mannheim
Interleukin - 6	$2x10^5$U/ml	1299972	Boehringer Mannheim
Serum-free Briclone	10ml	------	NCTCC
Linoleic acid	100mg	L1012	Sigma
$CaCl_2$	500g	C196/18/67	M and M
$CuSO_4$	500g	C8027 (lot 30H06135)	Sigma
$MnSO_4$	100g	M1144 (lot 80H03475)	Sigma
Na_2SiO_3	1L*	S1773 (lot 32H3454)	Sigma
NH_4VO_3	100g	A8175 (lot 29F06915)	Sigma
$(NH_4)_6Mo_7O_{24}$	100g	A7302 (lot 42H3506)	Sigma
$NiCl_2$	100g	N6136	Sigma
$SnCl_2$	100g	S8134 (lot 40H2513)	Sigma
$ZnSO_4$	100g	Z0251 (lot 40H07496)	Sigma

1L* 27% SiO_3 in 14% NaOH

2.5 CELL LINES

The cell lines used were anchorage dependent. All cells were obtained from the culture collection present in the laboratory. NRK cells are Normal Rat Kidney fibroblasts. These were originally supplied to this laboratory by Ian Pragnell from Beatson Institute, Scotland. CHOK1 are fibroblastic-like cells derived from Chinese Hamster Ovary. MDCK are epithelial-like cells derived from dog (Cocker Spaniel) kidney. Both MDCK and CHOK1 were supplied by the American Type Cell Culture. Table 2.5 lists the media in which each cell type was routinely grown.

Table 2.5 Routine Growth medium for cell lines

CELL LINE	BASAL MEDIUM	ADDITIVES
NRK	DMEM	5% FCS (# 41925), 1x L-Glutamine
CHOK1	Ham's F12	5% FCS (# 41925) or DHS (# 9120950)
SCC-9	ATCC	5% FCS (# 41925), 1x L-Glutamine, 0.4μg/ml HC
MDCK	DMEM	5% FCS(# 41925), 1x L-Glutamine

Abbreviations: HC = hydrocortisone; ATCC = 1:1 vol:vol DME:Ham's F12

2.5.1 Cell Growth / Maintenance

Cells were routinely maintained in the respective media described in Table 2 5 (8-9ml medium per 25cm^2 flask) The cells were incubated at 37°C in tissue culture grade flasks Cells were grown to confluency with feeding All fresh media used for feeding cells was incubated at 37°C before use All manipulations involving cells were carried out using aseptic techniques in a class 2 laminar flow Every item entering the laminar flow was swabbed in 70% Industrial Methylated Spirits (IMS) If dealing with several different cell lines, a waiting period of 15-20 minutes ensured no cross contamination of cell lines When large volumes of cells were required, cells were seeded into larger flasks (75cm^2 flasks) or roller bottles Where 2x10^7 or more cells were required, cells were grown in roller bottles with 100ml of growth medium and a minimum seeding density of 5x10^6 cells An initial rotation speed of 0 25rpm was used to allow the cells to attach and after 24 hours the rotation speed was increased to 0 75rpm on the roller bottle apparatus at 37°C

2.5.2 Cell Subculture Routine

When cells became subconfluent, flasks or roller bottles were trypsinized with a 0 25% trypsin versene (TV) solution The procedure described here is for a 25cm^2 flask but is the same for larger flasks and roller bottles Waste medium was removed from the cells The cells were washed in a pre-warmed TV solution, to remove any residual serum which contains enzymes that inhibit the action of trypsm 2ml of fresh TV was added The flask was incubated at 37°C for 5 - 10 minutes until the cells began to detach When a single cell suspension was attained, serum-containing medium was used to inactivate the trypsin The resulting solution was centrifuged at 1000rpm for 5 minutes The pellet was resuspended in fresh medium and counted Trypsin versene was prepared as follows

50mls of porcine trypsm was filter sterilized through a 0 22μm low-protein binding filter and added to 439mls of sterile PBS A 11mls of 1% EDTA solution brought the final volume to 500ml The TV solution was dispensed into 20 ml volumes and stored at -20°C

2.5.3 Subculture of cells in Serum-Free Medium

As no serum could be used to inactivate trypsm while passaging cells in SFM, trypsin inhibitor (TI) was made up such that a 1x volume would inhibit the action of 1x volume of trypsin Media was removed from cells and the cells rinsed in pre-warmed trypsin This was removed and 500μl TV was added per 25cm^2 flask, allowed to cover the entire surface and incubated until the cells began to detach (often less than 5 minutes for NRK and CHOK1 cells, but up to

10 minutes for MDCK cells depending on the extent of confluency). 500μl of pre-warmed TI was added into flasks and gently mixed. This was then removed to a universal. Fresh medium (about 4mls) was added into the flask to wash out any residual cells. This was added to the cell suspension and immediately centrifuged at 1,500rpm for 10 minutes at 4°C. All of the supernatant was gently removed and the pellet resuspended in 4ml of fresh medium. The cells were then re-centrifuged but only for 5 minutes. All the supernatant was removed and the cells resuspended gently in fresh medium. IMPORTANT: This process was always carried out as quickly and gently as possible to limit damage to the cells.

Trypsin inhibitor was made up as follows: 1.8g of TI will inhibit 1mg/ml trypsin.

The stock trypsin was 2.5mg/ml so 1.39mg/ml TI was required to inhibit when using equi-volumes. 100mg was diluted down into 7.2ml ATCC, filter-sterilized, aliquoted and stored at -20°C. These 10x stocks were thawed when required.

2.5.4 Cell Storage

Cells were routinely stored for indefinite periods of time in liquid nitrogen at -196°C. For each cell line, a master stock was kept in a separate culture collection. From the master stock, a working stock was obtained and this was kept separate from the master stock. Special care was taken with cell storage to ensure viability and absence of contamination when thawed down. The cryopreservative used in this laboratory is DiMethyl Sulf-Oxide (DMSO).

2.5.4.1 Cell Freezing Procedure

Cells to be frozen had to be in the log phase of growth. They were screened to ensure the absence of mycoplasma contamination. Cells to be frozen were routinely grown up in 75cm^2 flasks or roller bottles. Cells were trypsinized and resuspended in medium supplemented with 5% FCS. A viable cell count of at least 5x10^6 cells/ml was required (about 3-5ml from a 75cm^2 flask and 8-10ml from a roller bottle). The cryopreservative solution was made up with 10% DMSO and 50% FCS. The remaining 40% was medium supplemented with 5% FCS. 10ml stocks of the cryopreservation solution were made up and stored at -20°C.

Slowly, in a dropwise manner, the DMSO solution was added to the cells in a swirling motion. This step was very important as DMSO is toxic to cells. When added slowly the cells had a period to adapt to the presence of DMSO, otherwise the cells may have lysed. The suspension was then aliquoted into sterile cryovials. The cells were slowly frozen in the vapour phase of the liquid nitrogen for 3 hours and then stored in compartments in the liquid phase. Shortly after freezing, the viability was tested and usually found to be greater than 90%.

2.5.4.2 Cell Thawing Procedure

A vial of cells was removed from the liquid nitrogen and thawed quickly at 37°C Immediately upon thawing, the cells were transferred to a sterile universal containing serum-supplemented medium and centrifuged at 1000rpm for 5 minutes to remove DMSO which is toxic to cells at room temperature The cell stock was then resuspended in 8 - 9mls of growth medium and inoculated into a 25cm^2 flask

2.6 MYCOPLASMA ANALYSIS

Two methods were used to detect mycoplasma Routine mycoplasma analysis (every 6 to 8 weeks) was carried out by Una Gilvarry-Quigley or Cathy Halligan

2.6.1 Indirect Staining for Mycoplasma

In this method, conditioned media was collected from the cells to be tested and incubated with NRK cells previously shown to be mycoplasma negative After 5 days, the cells were stained with Hoechst 33258 (binds specifically to DNA) The presence of fluorescent bodies seen outside the cells was an indication of mycoplasma contamination Hoescht stain is a potential carcinogen and so was handled with due care

Procedure NRK cells, lower than passage 21 were inoculated at 1x10^5 cells/ml and grown on sterile cover slips in 30mm petri-dishes overnight The cells to be assayed had to be growing for at least 3 to 5 passages after thawing (to allow mycoplasma, if present to recover from freezing) Conditioned medium was obtained from the test cells and centrifuged at 1000rpm for 5 minutes 1ml of conditioned media was added to the NRK cells and allowed to incubate for about 5 days The waste medium was removed and cells were washed twice in PBS and once in a cold PBS/Carnoys (50/50) solution Immediately after this, the cells were fixed in Carnoys solution for 10 minutes (Carnoys fixative - Acetic acid Methanol-1 3) This prevented the cells from drying out The fixative was removed and the slides were air-dried After two washes in deionized water, 2ml of Hoechst 33258 stain (1 in 2000 dilution to 50ng/ml) was added The slides were stained for 10 minutes in the dark, as the fluorescence was slowly quenched when exposed to light After a further 3 rinses in deionized water, the slides were mounted in PBS A at pH 7 4 The slides were then examined under 400X and 1000X oil under a fluorescent microscope using a UV filter

2.6.2 Direct Culture of Mycoplasma

In this method, the conditioned medium from the test cells was inoculated into broths or agars

designed specifically to enrich mycoplasmic growth Mycoplasma Agar (CM401) and Broth (CM403) base are supplied by Oxoid To 9mls of sterile agar or broth base, the following supplements were added 2ml Horse serum, 1ml fresh yeast extract (0 25g/ml), 25μl penicillin and 130μl of 0 2% DNA (BDH,42026) Note Agar is first melted and then allowed to cool before addition of supplements

The test sample was then inoculated onto plates and into broth and allowed to incubate at 37°C for 3 weeks The plates were incubated in sealed CO_2 jars to prevent contamination Broths and plates were then checked regularly under a microscope for growth

2.7 MICROPIPETTE ACCURACY CHECKS

Accuracy checks were carried out on all micropipettes and multi-channel pipetters The accuracy and precision of 1000μl and 100μl pipettes were determined by standard methods involving repeatedly pipetting specific quantities of water and weighing them The specifications for these tests were supplied by Gilson

For multi-channel pipetters the above checks were carried out for the whole instrument (*i e* all 8 tip holders) together The suppliers (Brownes) recommended a precision check for the user and this was adapted to look at variation between individual 'channels'

The method involved aliquoting out a dye and reading the absorbances Stock neutral red (at 0 4%) was diluted down 1/10 in PBS and mixed The same volume was repeatedly pipetted onto a 96-well plate The results were then read at a dual wavelength of 570nm and 620nm on the ELISA plate reader The coefficient of variation (CV) was calculated as follows

% CV = (standard deviation) x 100 / mean For good user pipetting, the CV of the various columns should be less than 2 0% Between the 8 tip channels the CV should be less than 2%

2.8 GROWTH STIMULATORY ASSAYS

Growth stimulation assays were carried out using miniaturized assay systems to screen a wide variety of factors, while subculture assays were used to assess the effect of selected variables over a long period of time

2.8.1 Miniaturized Assay systems

Miniaturized assays were of two types, depending on plate size (24- and 96-well plate) For 24-well plate assays, results were determined by protein staining techniques or haemocytometer counts For 96-well plates, results were determined by cell counts, acid phosphatase activity or crystal violet dye elution The preparation of these assays was divided into two parts, sample

preparation and cell preparation This is demonstrated below in the preparation of a typical albumin screening assay

2.8.1.1 Sample Preparation

Stock components were made up in ATCC medium and diluted down as 200X or 1000X stocks of the final desired concentration and stored at -20°C The relevant stocks were thawed gently at 4°C and prepared as 100X stocks A 'Master Cocktail' was then made up of the appropriate components to give a final 2X sample This was made up on the day of the experiment The master cocktail contained all relevant factors, DHS (supplied by Flow), L-glutamine, albumins and lipids or Ex-cyte All assays were carried out without the addition of antibiotics or fungicidal agents

The 24-well plates were divided up so that each variable was set up in triplicate per plate with 8 variables per plate 0 5ml of each albumin solution was added to each of the triplicate wells The 96-well plates were divided so that each variable was set up with 8 repeats and 12 variables per plate Sample and control stocks were made up identically for 24- and 96-well plate experiments 0 5ml sample was aliquoted per 24-well plate and 100μl sample per 96-well plate Two controls were set up on every plate, a positive control of 5% FCS and a negative control The negative control was usually the background serum concentration or the SFM
A serial dilution curve of serum was included which was used to compare the extent of cell growth in experimental variables In Table 2 8 1, an example of making up an albumin dilution curve is given The final solutions are a 2X of the final desired concentration, so that when added to the cell suspension the albumin concentrations would be 0 5, 1 0, 2 0, 5 0 and 10mg/ml albumin with a 2% DHS background When all the variables were plated out, the plates were placed in a CO_2 incubator, for pH and temperature equilibration

Table 2 8 1 Sample preparation for setting up miniaturized assays

Sample label	Final 2X Albumin conc (mg/ml)	Stock 100mg/ml	ATCC ml	A ml	B ml	C ml	D ml	40% DHS (vol/vol) ml
A	20 0	2ml	7 0	--	--	--	--	1 0
B	10 0	--	4 5	5 0	--	--	--	0 5
C	4 0	--	8 1	--	6 0	--	--	0 9
D	2 0	--	4 5	--	--	5 0	--	0 5
E	1 0	--	1 8	--	--	--	2 0	0 2

2.8.1.2 Cell Stock Preparation

All cells used in growth stimulation assays were pretreated so that they were in the exponential phase of growth This was carried out by setting the cells up at a suitable concentration (1 x 10^5 cells per 25cm^2 flask) so that two to three days later, they were 50-70% confluent The cells were fed and the following day used in the experiment All experiments except where stated, were carried out three times, requiring three separate stocks of cells and test samples for each experiment

Cells were trypsinized as described in sections 2 5 3 and 2 5 4 depending on the assay system After centrifuging, the cells were resuspended in the appropriate basal medium Viable cell counts were carried out using trypan blue staining in duplicate If the coefficient of variation between counts was greater than 10%, an additional count was carried out

A master stock of cells was made up sufficient to carry out the entire experiment For 24- and 96-well plate experiments, the appropriate concentration for the various cell lines was used For 24-well plates, all cells were set up at 5x10^3 cells per well (*i e* 0 5ml of 1x10^4 stock cell suspension) For 96-well plates, final cell densities of 5x10^2, 8x10^2 and 1x10^3 cells per well were required for CHOK1 / MDCK, NRK and SCC-9 cells respectively

Cells were kept in a homogenous suspension by means of gentle inversion For 24-well plate assays, approximately 14ml aliquots were dispensed into sterile universals, one for each plate in the experiment For 96-well plate assays, 11ml aliquots were used Experience has shown that this method gave a more uniform result throughout the experiment
When all the cell suspensions had been aliquoted, a gentle mixing pattern (backward and forward, left to right) was used to evenly disperse the cells in the well
The plates were then placed on trays and covered in tin-foil (protection from contamination) The assays were incubated in 5% CO_2 at 37°C for 3 to 5 days until confluency was reached (depending on the cell line and assay conditions) Experiments were then taken down Results were obtained as described in 'Experiment End Points', section 2 9

2.8.2 Subculture Experiments

To study the long-term effect of selected factors, subculture experiments were required For serum-free systems, vented 25cm^2 flasks were used (due to uncontrollable increases in pH when MDCK cells were inoculated in a non-vented flask under serum-free conditions) Fresh media

was made up the day before use and sterility checked overnight to ensure no gross contamination had taken place (normally sterility checks are incubated for two weeks) Subculture experiments were incubated at 37°C and 5% CO_2 Media was made up so that all factors would be at 1X concentration

For MDCK and CHOK1 cells, an inoculation density of $2x10^5$ cells/flask was used 8ml of pre-warmed test media were added per flask The cells were incubated for 4 days The day before subculturing, 4ml of medium was removed and replaced with fresh medium Cells were trypsinized as described in section 2 5 4 When the cell number was determined, fresh flasks were inoculated with $2x10^5$ cells/flask Each variable was assayed separately three times

For NRK cells, serum-free subculture was carried out exactly as described above except that 6-well plates were used Subculturing in 6-well plates, reduced the cost of SFM (cost of FGF and PDGF) For 6-well plates, triplicate wells of two variables were assayed The wells were inoculated with $3x10^4$ cells per well (a total of 2ml per well) Each well was treated as totally separate, being trypsinized, counted and re-inoculated into fresh plates as separate variables As the depth of these wells was very low, these plates were always covered in parafilm to reduce evaporation of the media

2.9 EXPERIMENTAL END POINTS

2.9.1 Image Analysis

Image Analysis was used to measure cell area or colony number, on 24-well plates Crystal violet (BDH, 42555) was used to stain the cells Crystal violet was made up as a 0 25% solution in ultrapure H_2O and filtered before use This is a non specific protein binding dye For this reason, sufficient washing steps before staining, were carried out to remove serum proteins which could interfere in analysis

Waste media was removed and the wells were washed 3 times in PBS A (approximately 0 5ml per well) 0 5ml of 0 25% crystal violet stain was added to each well and left to develop for 5 - 10 minutes The crystal violet was then decanted and the plates washed with tap water to remove any unbound crystal violet The plates were left to dry before being analyzed using an image analyzer Crystal violet dye could be filtered and reused

2.9.2 Dye elution

After image analysis, the crystal violet dye was eluted from the plates, by adding 250μl of 33% glacial acetic acid to each well 100μl samples were aliquoted into a 96-well plate (2 samples from each 24-well plate) The absorbance was read on an ELISA plate reader at dual wavelength of 570nm and 620nm, 570nm is the wavelength specific for maximum absorbance of the crystal violet dye and 620nm is the wavelength which is specific for absorbance from the plastic in the plates themselves

2.9.3 Coulter Counts

Total cell counts were carried out using a ZR Coulter counter Cells were trypsinized in the usual way Where 24-well plates were involved, 0 5ml of TV was used to detach the cells 0 5ml of a single cell suspension was added to 19 5ml Isoton in a diluvial The diluvials were gently inverted and read on the coulter counter To calculate the cell number per well, the following was taken into account 0 5ml of cell sample was diluted into 20ml The coulter counter only measures the number of cells in 0 5ml This made the multiplication factor 40 For the 96-well plates 200μl was added per well The eight samples were then pooled and added to 18 4ml Isoton The multiplication factor for the number of cells per well was 3 125 This was determined as follows 1 6ml cell solution was diluted into 20ml as before the coulter only counts the number of cells in 0 5ml 8 wells were combined Multiplication factor = (20 x 2) / (8 x 1 6) = 3 125

2.9.4 Haemocytometer Counts

Plates were washed first m a small volume of TV This was totally removed 100μl was added per well for 24-well plates The plates were covered in parafilm to prevent evaporation of TV After 5 - 10 minutes incubation at 37°C, 50μl of serum-supplemented medium was added The solution in each well was pipetted up and down to ensure a homogenous cell suspension Cells were then counted on a haemocytometer Using a haemocytometer, cell counts are given per ml In the squares on the haemocytometer, the volume occupied by the sample is determined by the width (0 1cm) and the depth (0 01cm) So in the square, a total of 0 0001cm^3 or 0 0001ml is present When the number of cells in the well were counted, the count is divided by the volume (0 0001ml), to give the cell number per ml However, only 150μl volumes were present per well Results were then multiplied by 6 66 to give the cell count per well

2.9.5 96-well Acid Phosphatase Assay

The acid phosphatase assay is based on the activity of cellular acid phosphatases which can be related to cell number It was first developed by Connolly *et al* in 1985 An adaption of this method which was developed by Martin and Clynes (1991), was used in this study

For this procedure a para-nitro-phenyl phosphate buffer was required The buffer was made up with 0 1% Triton X-100 (Sigma, lot 119F0733) and 0 1M sodium acetate (Riedel-deHaen, 32318) at pH 5 5 This was stored at 4°C in the dark The substrate for the reaction para-nitro-phenyl (PNP) (Sigma, C104), was added just before use The complete PNP buffer was stored in the dark as it was light sensitive Waste medium was removed and the cells rinsed twice in 100μl PBS per well 100μl of freshly prepared PNP buffer (see above) was added to each well This buffer contained Triton X which lysed the cells and exposed cellular phosphatases to the phosphatase substrate The reaction was incubated in the dark at 37°C for 2 hours The reaction was stopped by addition of 50μl of 1M NaOH to each well The results were read on a Titertek plate reader at a dual wavelength of 405nm and 620nm, 405nm is the wavelength of maximum absorbance for the dye and 620nm is the wavelength specific for maximum absorbance of the plastic in the plates themselves

2.9.6 96-well Crystal Violet Dye elution

This was carried out essentially as described for 24-well plates except that a smaller volume (about 40 - 50 μl) of dye was required per well After incubation for 5 - 10 minutes, the dye was washed off the plates and were left to dry 100μl of 33% glacial acetic acid was added per well The plates were swirled to elute the dye into the acetic acid and the plates were then read as before

2.10 ALBUMIN LOADING EXPERIMENTS

Albumin loading experiments were carried out to see if the incorporation of fatty acids, cholesterol and phospholipids could improve the growth stimulating ability of fatty acid free albumin Two methods were employed, the Jager method and the Watt method

2.10.1 Method 1 (Jager *et al.*, 1988)

20μl of a freshly diluted stock of oleic acid (20mg/ml in ethanol) was slowly added per ml of fatty acid free-BSA (50mg/ml in PBS A) The mixture was then allowed to complex overnight at 4°C by gentle end over end rotation The mixture was then filter sterilized and stored at 4°C until use

Phospholipids and cholesterol were first dissolved in chloroform and then dried under a stream of nitrogen gas Lipids were then dissolved in ethanol and complexed in the same manner as oleic acid

2.10.2 Method 2 (Watt and Davis, 1989)

Lipids were dissolved in a few drops of chloroform at room temperature or in ethanol at 50°C in a 25ml glass beaker The solvent was then completely removed under a stream of nitrogen gas, leaving a film of lipid at the bottom of the beaker

10ml bicarbonate-free DME at pH 5 1 containing 1% delipidated albumin was added The beaker was immersed in ice and sonicated under air for 10 minutes at maximum energy (just below the foaming point) so that the lipids formed small micelles The solution was then filtered through a 0 22μm filter and stored at 4°C until use

2.11 BIORAD PROTEIN ASSAY

The BioRad Protein (Cat No 500-0006) Assay is a dye-binding assay in which albumin was used to construct the standard curve The protein content of test samples were then read from the standard curve

2.11.1 Procedure

Serial dilutions of BSA were made up in duplicate in sterile PBS A The concentration range was from 0 2 to 1 2mg/ml Test samples were diluted appropriately 0 1ml of either test sample or standard was added to clean labelled test tubes The dye was diluted 1 in 5 m PBS A and filtered through Whatman filter paper immediately before use 5ml of diluted dye was added per test tube The samples and standards were vortexed gently to form a homogenous solution After a period of 5 minutes to 1 hour, the OD was read at 595nm against a reagent blank The OD was plotted versus known concentrations and the unknowns were determined from the standard curve

2.12 AFFINITY CHROMATOGRAPHY USING HEPARIN-SEPHAROSE Cl-6B

Heparin Sepharose (HS) consists of a cross-linked sepharose matrix to which heparin is covalently linked using the cyanogen bromide method Heparin is a naturally occurring mucopolysaccharide which is polyaniomc in nature and thus reacts with many cationic compounds Heparin Sepharose is supplied by Pharmacia in a freeze-dried form (code no 17-

0467-01, lot no SL 208990) 1g HS powder gives about 4ml swollen gel To remove additives, the gel is washed on a sintered glass filter with about 200ml starting buffer per gram powder

2.12.1 Preparation of small-scale columns

The initial chromatography columns were run in 20ml capacity Econo-Pac disposable columns (cat no 732-1010) supplied by Bio-Rad 3 75g HS powder was allowed to swell for 15 minutes in the starting buffer (50mM Sodium Phosphate Buffer (Na_2HPO_4 or NaPi for short) at pH 7 4) NaPi was supplied by BDH (cat no 10245, lot 26218802) Instead of washing on a sintered glass filter, the swollen matrix was placed in one of the Econo-columns The matrix was then washed with small amounts of starting buffer, with occasional gentle inversion, until a total of 750ml was passed through the column The column was then stored at 4°C until use Indeed, once the sample had been applied to the column, all subsequent steps were carried out at 4°C

2.12.2 Application of sample

A 40ml solution of 15mg/ml BSA fraction V (Sigma cat no A4919, lot 100H04635) was made up in 50mM NaPi, pH 7 4 15-18ml of the BSA solution was added to a column that had been allowed to nearly run dry (the remainder was kept as the BSA control) The column was stoppered and gently inverted to form a homogenous solution The matrix/albumin solution was removed to a clean 100ml beaker As the matrix settled out of solution, some of the solution was aspirated off and used to rinse out the column The solution was then mixed gently over-night at 4°C

2.12.3 Step-wise Elution

The following day the mixture was transferred back into the column Some of the unbound fraction was used to rinse out the beaker and so, collect any residual matrix The unbound fraction was collected (approximately 18ml) and stored

The second step involved washing the column with 40ml starting buffer (50mM NaPi, pH 7 4) The first 8ml eluted (which was estimated to be the void volume) was added to the unbound fraction

The third, fourth and fifth steps involved elution at increasing salt concentrations The concentrations used were 0 5, 1 0 and 2 0M NaCl For the 0 5 and 1 0M NaCl, washes were

carried out as for the Buffer wash, with the first 8mls of each eluate being added to the previous one In the last step, 48ml 2 0M NaCl was used to wash out the column and the final volume was about 40mls After being analyzed for protein content (absorbance at 279nm), the samples were filter sterilized and kept at 4°C, until ready for diafiltration

2.12.4 Regeneration of column

The HS matrix is reusable once it is properly cleaned and stored The matrix was initially washed with 120ml 0 1M Tris-HCl, 0 5M NaCl, pH 8 5 The matrix was then washed with 160ml 0 1M sodium acetate in 0 5M NaCl at pH 5 5 Finally the column was washed out with a 20% (vol/vol) ethanol in ultra-pure water The column was sealed and stored in this solution at 4°C

2.12.5 Diafiltration of samples

The samples were diafiltered in order to replace the buffer in which the samples were collected with the growth medium required by the cells and to reduce the salt concentration The final salt concentration required for assaying was 0 02M NaCl, as is present in ATCC medium

2.12.6 Preparation of filter

Two types of filter were used, initially a YM5 (An 02361A) and then a YM3 (An 06177A) These Diaflo ultrafilters were supplied by Amicon The membranes were prepared for use by rinsing in ultra-pure water to remove the sodium azide, which is used as a preservative The filter was handled very gently to avoid scratching the glossy (skin) side The membrane was rinsed glossy side down in a large beaker of UP water for at least one hour, changing the water three times The filter was then mounted in the ultrafiltration cell and rinsed again by running 100ml UP water through the system under 50psi (provided by N_2 gas)

Before diafiltering a sample, all the pieces of the ultrafiltration cell were rinsed 3 times in UP H_2O The filter was placed glossy side up, so that it would be in contact with the medium The sample was applied, diluted to the appropriate volume (see Table 2 12) with ATCC medium at pH 7 4 (Table 2 12) Pressure of 50psi was applied to the ultrafiltration cell and the solution was continuously stirred The filtrate was collected and tested for protein content At the end of the filtration when about 15mls were left in the cell, the retentate was transferred to a 50ml sterile centrifuge tube The ultrafiltration cell was rmsed out with an appropriate volume of filtrate and added to the retentate to bring the final volume of retentate to the volume of the

original BSA solution applied to the column (about 18ml) The retentates were analyzed for protein concentration and filter sterilized

After each diafiltration, the filter and components were rinsed thoroughly with UP H_2O At the end of the diafiltrations, the membrane was soaked three times within an hour in UP H_2O The membrane was then stored in a 1 5M NaCl solution at 4°C until required again

Table 2.12 Dilution factors for diafiltration of fractions

FRACTION	INITIAL -> FINAL VOLUME	DILUTION WITH BASAL MEDIUM	DILUTION
Unbound Fraction	18ml	18ml F + 72ml BM ->18ml	1/5
Buffer Wash	40ml -> 18ml	18ml CF + 72ml BM -> 18ml	1/5
0 5M NaCl Wash	40ml F -> 18ml(CF)	18ml CF + 72ml BM -> 18ml(CF1) 18ml CF1 + 72mlBM -> 18ml	1/25
1 0M NaCl Wash	40ml F -> 18ml(CF)	18ml CF + 162ml BM -> 18ml(CF1) 18ml CF1 + 72ml BM -> 18ml	1/50
2 0M NaCl Wash	40ml F -> 18ml(CF)	18ml CF + 162ml BM -> 18ml(CF1) 18mlCF1 + 162ml BM -> 18ml	1/100
Control Albumin	18ml	18ml BSA + 72ml BM -> 18ml	1/5

Abbreviations BM = Basal Medium, F = Fraction, CF = Concentrated fraction, -> = diafiltered down to a certain volume

2.12.7 Protein determination

Samples of all retentates, filtrates from the initial concentration step in Na_2HPO_4 were kept for protein analysis, as were samples of retentates and filtrates concentrated in ATCC medium Quartz cuvettes were used 2 9ml sterile PBS A was aliquoted into each cuvette 100μl sample was added and the cuvettes were gently inverted using parafilm The blank for samples in ATCC medium was 100μl ATCC in 2 9ml PBS A Samples m Na_2HPO_4 buffer were read against a blank containing 100μl NaPi and 2 9ml PBS A The spectrum was read from 600nm to 240nm The protein extinction coefficient at A_{279} is 0 667 A sample calculation is given

Absorbance at peak at 279nm = 0 200, Background absorbance at 600nm = 0 001

Dilution Factor = 100μl in 3ml => 30,

Protein concentration = (A_{279} - A_{600}) x Dilution factor / Extinction coefficient

= (0 200 - 0 001) x 30 / 0 667

= 8 95mg/ml

For the 0 5, 1 0 and 2 0M NaCl fractions, either neat or 1 in 2 dilutions of the samples were made The controls varied accordingly

2.12.8 Large-Scale Columns

To obtain larger volumes of each fraction, the column was scaled up to make use of the Biopilot The Biopilot is an automated liquid chromatography system supplied by Pharmacia (Code No 18-4600-06) The Biopilot consists of a liquid column chromatography controller, pH, conductivity and UV monitors, two pumps, a fraction collector and a variety of columns types Programs were designed to control the sequential elution of buffers

The preparations and applications of a sample to the matrix were as described above with the exception that everything was scaled up approximately 2 5-fold All buffers were filtered and degassed to ensure no solids could clog the small bore tubing used on the Biopilot

10g HS matrix was allowed to swell 1 5L ultrapure H_2O was used to rinse the column, followed by 500ml starting buffer (50mM NaPi at pH 7 4) 87 ml of a 15mg/ml BSA solution was made up and 53ml were mixed gently with the matrix overnight

Due to the larger volume of buffer required for the elution, the process was split up over 2 days On the first day, all fractions except the 2 0M NaCl were run 2 0M NaCl at 4°C over a period of time will form crystals which could block the small bore tubing in the Biopilot For this reason the last wash with the 2 0M NaCl was left for the following day The 2 0M NaCl buffer was left at room temperature overnight, to prevent the formation of solids 120ml of each of the NaCl buffers were used in the separation Diafiltration was carried out as described above except that the volumes were initially concentrated to 25ml 20ml was taken for diafiltration and where previously 72ml ATCC had been added, now 80ml ATCC was added

2.13 SDS-PAGE

The Laemmli method (Laemmli and Favre, 1973) was used The system described here is based on running two vertical gels (about 20ml total volume) Stock solutions include

1 Stock Acrylamide 30% acrylamide (Riedal-deHaen, 62021), 0 8% NN-bis-methyl-acrylamide (Sigma, M2022)

2 Stock Tris-HCl 1 5M Tris-HCl (Sigma 7-9, T1378), pH 8 8

3 Stock 10% SDS (Riedal-deHaen, 62862)

4 Stock Tris-HCl 0 5M Tris-HCl, pH 6 8

5 Tank Buffer 0 05M Tris-HCl, 0 384M glycine (Riedal-deHaen, 33226), 0 1% SDS (2X used by Laemmli and Favre)

6 10% (w/v) Ammonium persulphate (LKB Bromma, 1820-103) (freshly made just before use)

7 0 1% (w/v) Bromophenol blue (LKB Bromma, 1840-901)

2.13.1 Preparation of Resolving Gel

Before making up the resolving gel, it was necessary to ensure that all stocks were present and to clean the gel apparatus (cleaned with water and wiped down with methanol) The following mixture was enough to make up 2 gels

15ml stock acrylamide

11 25ml stock Tris-HCl, pH 8 8

0 45ml 10% SDS

17 80ml Ultrapure H_2O

The solution was gently mixed To this was added in order 45μl TEMED (Aldrich T2,250-0) and 450μl ammonium persulphate The solution was degassed and about 15ml was applied per gel A 0 1% SDS solution was made up and used to level out the top of the resolving gel Although the gel normally set within 45 minutes, it was usually left overnight

2.13.2 Preparation of Stacking Gel

Before applying the stacking gel, the 0 1% SDS solution left on top of the resolving gel overnight was removed and rinsed with ultrapure H_2O The stacking gel was made up as follows

2ml stock acrylamide

2 5ml stock Tris-HCl, pH 6 8

0 1ml 10% SDS

5 29ml Ultrapure H_2O

To this was added 10μl TEMED and 100μl 10% ammonium persulphate The solution was degassed for 40 seconds and applied on top of the resolving gel The combs were put in place carefully to avoid creating any bubbles The stacking gel took about 3 hours to set

When the gels were set, the combs and the gaskets around the edge of the gel plates were removed The plates were carefully placed in the tank ensuring that no bubbles were present under the gels Once secured, sufficient tank buffer was applied to cover the tops of the gels The samples could then be applied to the gel

2.13.3 Sample preparation

The sample buffer was made up as follows 20ml ultrapure H_2O, 12 5ml stock Tris-HCl, pH 6 8, 10ml 10% SDS (Check pH is 6 8), 5ml glycerol, 0 5ml 0 1% bromophenol blue This was brought to 50ml with ultrapure H_2O Samples were diluted down to 1mg/ml solution in ultrapure water (Note Where necessary samples were desalted) 50μl of the sample was added to 4 7ml sample buffer with 0 25ml mercaptoethanol (Sigma M7522) (in fume hood) 0 3ml of this sample was boiled for 2 minutes (in fume hood) 20 to 40μl samples were applied to the gel The standard mixture, SDS-7 was supplied in a vial (Sigma, Daltonmark VII-L™) Contents were dissolved in 1 5ml sample buffer (1mg/ml) and prepared in the same manner as the sample Gels were set to run at 30mA (constant amperage) After the first half hour, this was increased to 35mA

2.13.4 Silver Stain for protein detection on SDS-PAGE gels

The gels were stained using the method of Oakley *et al* (1980) The glass plates were removed and gels were fixed in a 10% w/v gluteraldehyde solution (Sigma, G6257) for 30 minutes (in fume hood), washed 3 times in ultrapure H_2O and left shaking overnight in ultrapure H_2O Gels were then immersed in a freshly prepared silver solution (Table 2 13) After incubation at room temperature with gentle shaking, gels were washed 3 times in ultrapure H_2O and moved to a clean container Freshly made developing solution (Table 2 13) was then added As soon as staining was observed, the gels were removed from the developing solution and washed 5 times in ultrapure H_2O to stop the reaction Gels could be stored for prolonged periods in a solution containing 20% glycerol, 10% methanol, 7% acetic acid in ultrapure H_2O

Table 2.13 Silver Stain procedure

STAINING SOLUTION	DEVELOPING SOLUTION
Immediately before use mix in this order 33ml 0 36% NaOH 2 1ml ammonia (BDH AnalaR, 100125S) 6ml $AgNO_3$ (BDH, 10223) freshly made up) 109ml ultrapure H_2O	Immediately before use, mix in this order 250μl citric acid (freshly made up) 125μl formaldehyde (BDH AnalaR, 10113) 250ml ultrapure H_2O

Ammonia and formaldehyde were taken from stock bottles Their addition and subsequent additions took place in a fume hood

2.14 LYOPHILIZATION

Lyophilization of samples was carried out using a 4 5L Consol freeze dryer (Vitris) Samples were desalted if necessary (section 2 15) and frozen at -20°C on a slant in 25ml universals, to expose maximum surface area Samples from reverse phase-HPLC which contained acetonitrile

and trifluoroacetic acid were frozen at -70°C The lids of the universals were removed and parafilm punctured with tiny holes was used to cover the tops of the universals The freeze drying cycle generally lasted for 35 hours For samples containing solvents, a solvent trap, based on liquid nitrogen was used to remove harmful solvents from the vacuum before they reached the pump

2.15 DESALTING OF SAMPLES

Desalting of samples was carried out using Econo-Pac™ columns The columns were disposable desalting columns (Biorad, cat no 732-2010) with a gel volume of 10mls The packing material was supplied fully hydrated in 10mM $NaHPO_4$, 10mM NaCl, pH 7 0 and 0 02% NaN_3 and was covered by a frit to prevent the gel from drying out The columns were first washed with ultrapure H_2O to remove the storage buffer The H_2O was allowed to run completely through the columns 3ml of sample was applied and allowed to run through the column This was discarded and the sample eluted from the column with 4ml ultrapure H_2O

2.16 DIALYSIS

Dialysis was carried out using tubing with a 10,000 molecular weight cut off membrane Dialysis tubing was boiled in a 10mM EDTA solution at pH 7 4 for 5 minutes and washed through with ultrapure H_2O three times The tubmg was then left soaking overnight in ultrapure H_2O Dialysis tubing was double knotted and the samples were applied Dialysis was allowed to continue overnight at 4°C with gentle mixing in sufficient ATCC medium
Samples were then removed, filter-sterilized and assayed for bioactivity

2.17 REVERSE PHASE - HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Reverse phase-High Performance Liquid Chromatography (rp-HPLC) was carried out on the basis of the work described by Congote (1987) This involved running a mobile phase of 20 - 80% acetomtrile (ACN) and 0 1% trifluoroacetic acid (TFA) in ultrapure H_2O through a C18 column A linear gradient of 20 - 80% ACN was used for 40 minutes at 2 5ml/min (Congote) However, due to the smaller size of the column used in these experiements and to limitations in the HPLC equipment (Waters Model 660 solvent programmer, Model 510 Mixer, Lambda-Max (Model 481) LC Spectrophotometer), the gradient was run for 26 minutes at 1ml/min A pre-guard column (Guard-Pak™ μBondapak™ C18, P/N 88070) was placed in front of the column

2.17.1 Solvents

Acetonitrile (Labscan HPLC grade, C2502), methanol (Labscan HPLC grade, C2517) and trifluoroacetic acid (Aldrich 29,953-7) were HPLC grade reagents Stock TFA was kept under N_2 gas A 10% working stock was made up in ultrapure H_2O and kept in the dark at 4°C

2.17.2 Column efficiency

The column used was a Waters μBondapak CN-phenyl C18 column, P/N, code 27324 with dimensions 3 9mm x 300mm in a steel column The column efficiency was tested according to the 5-Sigma method as recommended by Waters The plate count measurement provides a numerical check for the quality of the column To determine this, a sample of acenaphtene (Aldridch, lot 3954g) at 0 5mg/ml in 60/40 ACN/H_2O was applied to the column calibrated in 60/40 ACN/H_2O at a flow rate of 1 5ml/mm The UV detector was set up at 254nm with an attenuation of 0 05 Aufs The resulting bell shaped curve was used to determine N, the number of plates

$$N = 25\ (Vr/W)^2$$

where Vr = volume to the apex of the peak (ml)

W = volume at 4 4% of the peak height (ml)

The value of N was found to be 4,039 For this column, at a flow rate of 2 5ml/min, the minimum number of plates per column should be 3,000

2.17.3 Priming System

When priming the HPLC system, samples of 20% and 80% ACN were run through the system to ensure no background noise existed Stock solutions of 20% and 80% ACN and 0 1% TFA in ultrapure H_2O were prepared by filtering through 0 22μm HPLC filters (Millipore HPLC filters, HVLP04700) and degassing the solvents for 15 - 20 minutes Two pumps were used, pump A connected to the 20% ACN reservoir and pump B connected to the 80% ACN reservoir 20% ACN was run through the column *via* both A and B pumps (initially containing 20% ACN) for 10 minutes at 0 8ml/min Pump B was filled with 80% ACN and then run through the mixing chamber for 5 minutes The mixing chamber was cleaned out with 20% ACN from pump A for a further 5 minutes Pump A was run at 1ml/min The system was now primed and ready for sample application

2.17.4 Sample Preparation

Samples of BSA or the 0 5M NaCl fraction to be loaded onto the column were made up in a stock solution of 400mg/ml BSA (for 0 5M NaCl fraction, 20mg/ml) in 10% ACN and 0 1% TFA in ultrapure H_2O These solutions were filtered through HPLC filters and kept on ice until injection As the injection loop only had a capacity for 20μl, any number from 12 to 20 runs were required to get a sufficient sample concentration to test for bioactivity

When the sample was injected, the gradient program and the UV chart recorder were started simultaneously The exhaust from the column was connected up to a fraction collector which collected 2ml fractions These fractions were transferred to a plastic universal, labelled and frozen on a slant at -70°C These fractions were subsequently lyophilized to remove ACN and TFA which would interfere in growth stimulatory assays

Protein concentration was monitored at 280nm by a UV spectrophotometer and recorded on a chart recorder running at 5mm/min with varying detection range (usually 0 - 2 Aufs) When Coproporphyrin (Sigma, cat no COP-1-5) was run as a control at 0 2mg/ml to determine the elution position of erythrotropin-like factor, the detection was set at 440nm

2.18 STABILITY STUDIES

2.18.1 Trypsin digestion

Trypsin digestion was carried out by incubating a 15mg/ml BSA solution for 2 hours at 37°C with 10μg/ml trypsin The digestion was stopped by addition of sufficient trypsin inhibitor to inactivate the trypsin

2.18.2 Pepsin digestion

Pepsin digestion was carried out as described by Polet and Spieker-Polet (1975) Albumin was digested with pepsin at a concentration of 1 - 2% of the total protein for 1 hour at 37°C at pH 2 6 With 1 5ml of 10mg/ml BSA, 100μl of 0 3mg/ml pepsin was added The pH was gradually reduced to 2 6 by addition of 10μl aliquots of 1M HCl After incubation, the digestion was stopped by bringing the pH up gradually to 7 4 by 10μl additions of 1M NaOH

2.18.3 pH stability

Samples of BSA or 0 5M NaCl fraction were dispensed out into universals The pH of each sample was adjusted by small additions (10μl at a time) of 1M HCl or 1M NaOH, gently mixing and reading the pH After 2 hours incubation at 37°C, the pH was readjusted The total

volume of acid or base added was recorded, and all the samples were brought up to the same concentration before filter-sterilizing and assaying

2.19 ANALYTICAL ASSAYS

Biochemical determination kits were used for the determination of citrate, fatty acid and phospholipid content of the fractions obtained from affinity chromatography of BSA on a heparin sepharose column All fractions were at a concentration equivalent to 25mg/ml BSA Procedures for determinations were carried out as described in each kit

2.19.1 Fatty acid determination

Fatty acid determination was based on the measurement of non-esterified fatty acids (Boehringer Mannheim, cat no 1,383,175) The principle behind detection was based on the conversion of free fatty acids to acyl-coenzyme A (acyl-CoA) in the presence of acyl-CoA synthetase and ATP The resulting acyl-CoA was oxidized in the presence of acyl-CoA oxidase to produce H_2O_2 which reacted with a dye to produce a colour complex measured at 546nm The linearity was up to 1 5mM free fatty acids

2.19.2 Phospholipid analysis

Phospholipid analysis was carried out using an enzymatic determination kit (bioMerieux, Phospholipids enzymatiques PAP 150, cat no 6,149,1) which measures the presence of lecithin, lysolecithin and sphingomyelin The measurement was based on the hydrolysis of phospholipids by phospholipase D to yield choline Choline was then used as the substrate for a colour change measured at 505nm The linearity was between 0 - 10mM

2.19.3 Citrate Determination

An enzymatic UV determination kit supplied by Boehringer Mannheim (cat no 139076) was used determine the level of citrate in samples The method was based on the conversion of citrate to oxaloacetate (catalysed by citrate lyase) The oxaloacetate and its decarboxylative product, pyruvate were reduced The amount of NADH consumed in the reduction was used as the basis of determining the amount of citrate present (absorbance at 340nm)

2.20 GEL FILTRATION

Gel filtration was carried out using sephacryl 200 packing material The system was automated by use of the Biopilot

2.20.1 Packing and calibration of column

A 70% slurry of S200 (Pharmacia, S200 High Resolution, 17-0854-01) in ultrapure H_2O was made up The slurry was mixed to a homogenous solution The slurry was allowed to settle for 30 minutes after which a certain volume of the supernatant was removed (taking out 'fines') The slurry was brought back up to a 70% consistency The slurry was carefully applied to a clean column (Pharmacia, XK16/100) using a packing program to ensure an even gel This took several attempts to get a suitable volume of gel bed (*i e* it was necessary to make sure that there was no dead space between the top of the gel and the inlet port)

The column was calibrated by applying a sample of filtered blue dextran (2mg/ml in Na_2HPO_4) *via* the super loop and running the column The exhaust tubing was connected to a fraction collector set up to collect 2ml fractions As the blue dextran migrated down through the column it was monitored visibly A sharp blue front was seen with a slight tail This remained the same all the way through the column Due to the high molecular weight of blue dextran, it was used as a marker to indicate the void volume of the column (Vo) This was determined by obtaining the 'bluest ' fractions and looking at the absorbance spectrum The maximum peak was found to be at 615nm so this was chosen to analyze other fractions around the most coloured fraction A single sharp peak was observed when the absorbance at 615nm was plotted against fraction number

A standard curve was prepared by running the samples shown in Table 2 20 1 and measuring the absorbance at 280nm By running these standards a value for the elution volume (the fraction of maximum protein absorbance (Ve)) was obtained When the log of the molecular weights of the markers were plotted against Ve/Vo, a linear relationship was obtained This allowed the identification of the molecular weight of a sample once the elution volume was known

Table 2.20 Gel filtration molecular weight markers

PROTEIN	SIZE	CAT NO	CONC	MOLECULAR WEIGHT
ß-Amylase	15mg	A8781 (lot 105F8680)	4mg/ml	200,000
Alcohol Dehydrogenase	25mg	A8656 (lot 78F9405)	5mg/ml	150,000
Bovine Serum Albumin	50mg	A8531 (lot 68F9402)	10mg/ml	66,000
Carbonic Anhydrase	15mg	C7025 (lot 107F9345)	3mg/ml	29,000
Cytochrome C	10mg	C7150 (lot 105F8670)	2mg/ml	12,400

2.20.2 Sample application

Samples were applied at 50mg/ml BSA (or equivalent protein concentration of the 0 5M NaCl fraction that would be present in the untreated albumin) As phenol red could specifically bind to albumin and interfere with absorption readings, a phenol red-free DME (Gibco, 11880-028) was used as the mobile phase

Due to the dilution effect when the sample was applied to the column (approximately 1 in 5), the fractions would be at 10mg/ml and ready for assaying in low serum medium For low serum assays, cells were set up in Ham's F12 (including serum, L-glutamine and sodium pyruvate), thus when combined, the basal medium would be ATCC For serum-free assays, dilutions were made in McCoys 5a medium (final concentration 0 1mg/ml) Two types of serum-free assays were set up depending on the serum-free constituents The simpler of the two contained 10μg/ml insulin and 1 39μg/ml Fe_2SO_4 The complex SFM in addition contained 1ng/ml ß-FGF and 5ng/ml PDGF

2.21 DIAFILTRATION EXPERIMENTS

Sequential diafiltration experiments were conducted using the same procedure as described in section 2 12 Diafiltration membranes of 100, 30, 10 and 3kDa cut-offs were used A stock solution of BSA was made up at 20mg/ml (150ml in total) 145ml of BSA in ATCC was diafiltered using the 10kDa membrane The 10x retentate (14 5ml) was kept for assaying 130ml of the filtrate was then diafiltered using the 30kDa membrane The procedure is shown in Table 2 21

Table 2.21

Membrane Cut-Off (kDa)	Initial Volume (mls)	Retentate Volume (mls)	Filtrate Volume retained (mls)
100	145	14 5	15
30	115	11 5	25
10	75	7 5	15
3	50	5 0	45

The retentates were then at a 10x solution which had to be diluted to assay at 10mg/ml The filtrates were at 20mg/ml (or equivalent to 20mg/ml BSA) and were diluted 1 in 2 before assaying

2.22 EXTRACTION

Extraction of albumin with organic solvents was based on the procedure used by Tigyi and Miledi (1992) Each extraction involved three separate extractions using the same sample of albumin and combining the organic phase from the three extractions For each 100mg protein, 1ml of organic solvent was used The organic solvents used were methanol (Labscan HPLC grade C2517), ethanol (Merk, 986 2500), chloroform (BDH AnalaR, 10077), acetone (BDR AnalaR, 10003) and di-ethyl-ether (BDH AnalaR, 10094)

1ml of each solvent was added to 100mg of crystalline BSA (cat no A4919, lot 110H-04635) in a glass universal and allowed to stir very vigorously at room temperature for 1 hour Due to the rate at which di-ethyl-ether and chloroform evaporate, all universals were covered in parafilm Each solution was then transferred to an eppendorf and centrifuged down for 10 minutes at 5,000 - 10,000g in a Biofuge

Supernatents were removed to a fresh glass universal and the pellet was resuspended in 1ml fresh solvent This procedure was repeated two more times All the supernatents from each extraction were pooled together and the solvent was evaporated off under stream of N_2 gas in a fume hood The pellets were resuspended in the appropriate solvent and also dried under a stream of N_2 gas All solvent and protein residues were reconstituted into 2ml ATCC (50mg/ml albumin) and biological activity was assayed at 5mg/ml Dilutions were based on the initial protein concentration being 100mg

2.23 THYMIDINE INCORPORATION ASSAY

The ability of samples to induce uptake of exogenous thymidine, an indication of DNA synthesis was measured by assessing [^{3}H]-thymidine incorporation into the DNA under continuous labelling conditions **NOTE** Great care was taken when handling radio-active material At all times when handling plates immediately before or after addition of ^{3}H-thymidine, two pairs of gloves were worn and work was carried out in a well labelled, protective hood University guide lines were followed during use and disposal of cells and waste containing ^{3}H-thymidine

The assay procedure involved setting up NRK cells at $2x10^4$ cells/well in 96-well plates As these cells were in SFM (McCoys 5a + insulin and Fe_2SO_4), the cells did not require to be starved of serum factors After 18 - 24 hours, the medium was removed Fresh medium and the factors to be tested (100μl) were added. Approximately 4 hours later, 5μl thymidine was

added (100μCi) to each well

The plates were incubated for a further 24 hours after which the assay was harvested Waste media containing ^{3}H-thymidine was removed to a beaker containing RBS to deactivate the thymidine The plates were washed once in PBS A The waste was again removed to a beaker containing RBS 200μl 1M potassium hydroxide was added per well to lyse the cells The plates were incubated at 37°C for 1 hour

After the contents of each well were thoroughly mixed, 100μl aliquots per well were added to 5ml scintillation fluid (Ecolite ICN cat no 882475) The disintegrations per minute were counted using a scintillation counter

2.24 STATISTICAL ANALYSIS

Statistical analysis was carried out as follows For assays in which N (the number of wells per variable) was greater than or equal to 3, the average and standard deviation was obtained according to the equation (Freund, 1979)

$$s = ((n(\Sigma x^2) - (\Sigma x)^2) / n(n-1))$$

where s = standard deviation
n = number of variables
x = numerical values

For most experiments, each assay was repeated 3 times The average and standard deviation of these three separate assays were expressed as the average percentage growth relative to the growth obtained by a control on each plate This meant that for every variable, there were three separate values of the average and standard deviation A standard error of the mean was not used in general as this would not take into account the deviation of the control As growth in low serum and serum-free medium could result in variable growth per assay, the results for three different assays were given and in some instances a graph of the assay that was most representative of the results obtained Where a standard error of the mean was obtained (see sections 3 3 and 3 4), the deviation from each variable was not taken into account

When there were only 2 wells per variable, the average of the two results was given with the actual results (as a percentage growth relative to a control) in brackets

3.1 GROWTH OF NRK IN LOW SERUM AND SERUM-FREE MEDIUM

Most references citing a serum-free medium (SFM) for normal rat kidney (NRK) cells or NRK subclones use the SFM described by Rizzino in 1984 for NRK clone 49F cells (see Table 3.1). Several attempts at growing parental NRK cells (as opposed to the subclone NRK-49F) in the SFM designed by Rizzino (1984) failed in our hands. No requirement for attachment factors was seen by Rizzino when the cells were grown on plastic. However, as the cells did not proliferate, changes were made to include attachment factors (1.33μg/cm^2 laminin, 1.5μg/cm^2 fibronectin). These attachment factors had been used by Rizzino, who found that both factors were equally good over a short time but laminin was found to be superior over an extended time. The SFM was tried with each of the factors missing individually. In all cases the cells attached but within 48 hours the cells became spindle-like in shape and appeared to peel off the plates.

As the SFM of Rizzino (1984) did not support the growth of NRK cells, it was decided to try and develop a SFM by first looking at the effect of a variety of components in a low serum medium. For this, Donor Horse Serum (DHS) was chosen. DHS was selected as the growth achieved with 5% DHS was not quite as good as that achieved by good FCS batches, so added stimulatory factors might be more readily identified. By using low concentrations of serum and testing the factors commonly used in SFM, it was hoped to identify some of the factors that may be required by NRK cells in serum-free medium. 2% DHS was chosen arbitrarily.

Table 3.1 Serum-free medium designed for NRK and subclones

AUTHOR	RIZZINO (1984)	NEWMAN (1986)	NUGENT (1989)
Insulin (μg/ml)	10	5	15
Transferrin (μg/ml)	5	5	5
EGF (ng/ml)	10	10	10
Lipoprotein (μg/ml)	300	100	100
Laminin (μg/cm^2)	12.5μg/ml	5μg/ml	1.25
FGF (ng/ml)	150	50	50
BSA-linoleic acid (mg/ml)	---	0.5	0.5
Others	25nM Na_2SeO_3 10ng/ml PDGF	5nM Na_2SeO_3	10nM Na_2SeO_3 100ng/ml Retinoic acid 2-5μg/ml fibronectin

All serum-free media were based on ATCC as the basal medium and were designed for NRK subclones 49F

3.1.1 EFFECTS OF INSULIN, TRANSFERRIN, BSA AND EX-CYTE ON NRK CELLS

The most common additives in serum-free medium described in the literature were found to be albumin, insulin and transferrin and some lipid source The ranges of concentrations for these components used were, 0 5 - 2 0mg/ml bovine serum albumin (BSA), 1 - 10μg/ml insulin (bovine pancreas) and 1 - 10μg/ml transferrin (human transferrin) It was decided to test the most common factors over a range of concentrations with 2% DHS background For the lipid source, a commercial lipoprotein preparation called Ex-cyte (Pentex product, supplied by Miles Diagnostics) was used Growth stimulatory assays were carried out in 24-well plates and analyzed using image analysis (section 2 9) The results are shown in Figures 3 1 1 1-4

3.1.1.1 BSA

Figure 3 1 1 1 shows BSA fraction V to be stimulatory The stimulatory effects were seen at lower concentrations (0 5 - 2 5mg/ml) with a maximum stimulation of about 2-fold over the control (2% DHS only) At higher concentrations, the BSA either lost its stimulatory effect or became inhibitory At concentrations of 10mg/ml the cells appeared to be peeling off the plates

3.1.1.2 Insulin

The results for insulin showed the highest stimulation of the four components tested (Figure 3 1 1 2) Stimulatory activity was seen in the microgram range with maximum growth at 20μg/ml, the highest concentration tested Results varied between three separate experiments but maximum stimulation was about 5-fold to 8 5-fold over the control (2% DHS) Unlike BSA, no inhibition was seen at higher concentrations

3.1.1.3 Transferrin

The results for bovme transferrin are shown in Figure 3 1 1 3 The levels of stimulation were slightly lower than those obtained with BSA Maximum growth appeared at concentrations of 2 5 to 10μg/ml Above 10μg/ml, transferrin became less stimulatory

3.1.1.4 Ex-cyte

Ex-cyte results (Figure 3 1 1 4) showed consistently better stimulation at the higher concentrations tested (20 - 40μg/ml) A plateau effect was seen at 30 - 40μg/ml with maximum activity of between 1 6-fold and 2 7-fold stimulation over the control depending on the assay

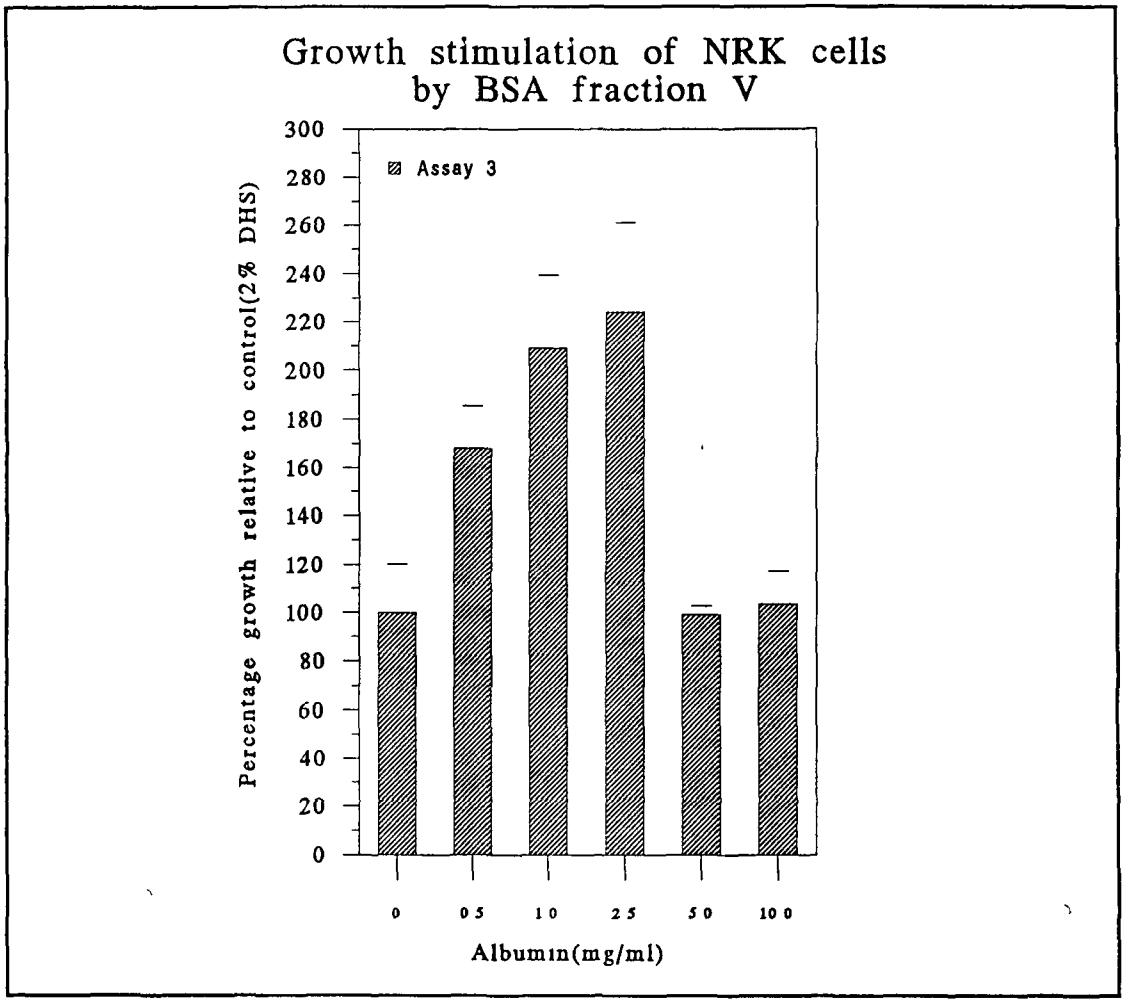


Figure 3.1.1.1 shows the growth response of NRK cells to BSA fraction V in low serum medium. The results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as the end point and the results for 3 separate experiments are shown in Table 3 1 1 1

Table 3.1.1.1 Growth stimulation of BSA fatty acid free (mg/ml)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
2% DHS	100 0 ± 11 2	100 0 ± 7 08	100 0 ± 20 2
+ 0 5 mg/ml	184 1 ± 15 2	227 0 ± 14 5	167 0 ± 17 7
+ 1 0 mg/ml	143 0 ± 3 90	275 4 ± 18 7	209 0 ± 30 4
+ 2 5 mg/ml	120 0 ± 15 2	289 6 ± 24 0	224 0 ± 36 7
+ 5 0 mg/ml	84 30 ± 8 00	86 20 ± 26 9	101 0 ± 13 8
+ 10 mg/ml	51 10 ± 17 2	112 1 ± 3 50	103 0 ± 13 6

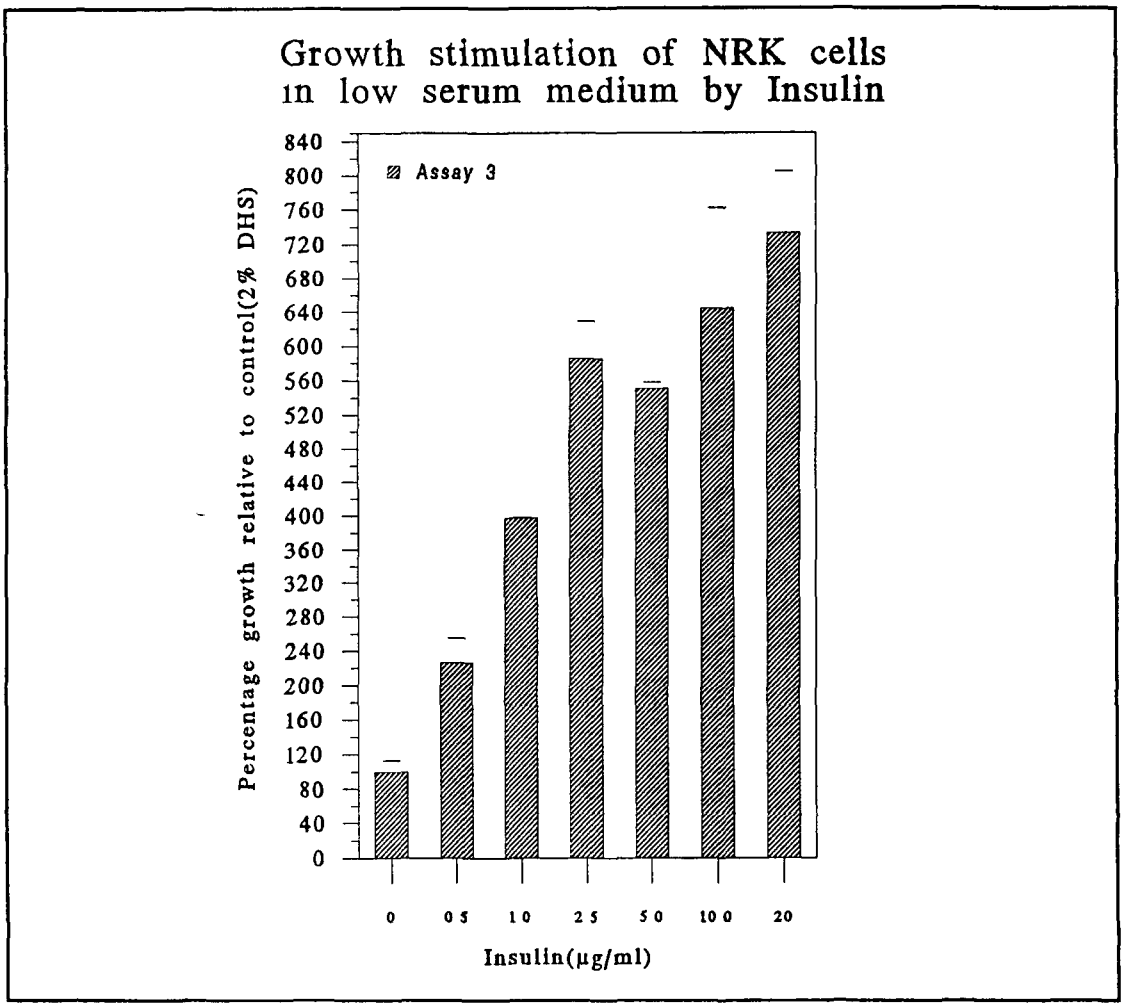


Figure 3.1.1.2 shows the growth response of NRK cells to bovine insulin in low serum medium The results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as the end point and the results for 3 separate experiments are shown in Table 3 1 1 2

Table 3.1 1.2 Growth stimulation of insulin (µg/ml)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
2% DHS	100 0 ± 0 34	100 0 ± 9 00	100 0 ± 12 8
+ 0 5 µg/ml	354 9 ± 70 9	89 10 ± 11 1	226 2 ± 28 8
+ 1 0 µg/ml	487 0 ± 47 9	--------	397 9 ± 0 64
+ 2 5 µg/ml	774 9 ± 162 2	354 4 ± 55 4	585 3 ± 43 3
+ 5 0 µg/ml	702 2 ± 53 7	454 5 ± 52 0	551 2 ± 6 07
+ 10 0 µg/ml	678 2 ± 17 0	430 0 ± 63 1	643 9 ± 117 4
+ 20 0 µg/ml	858 8 ± 15 4	496 0 ± 10 9	732 9 ± 71 5

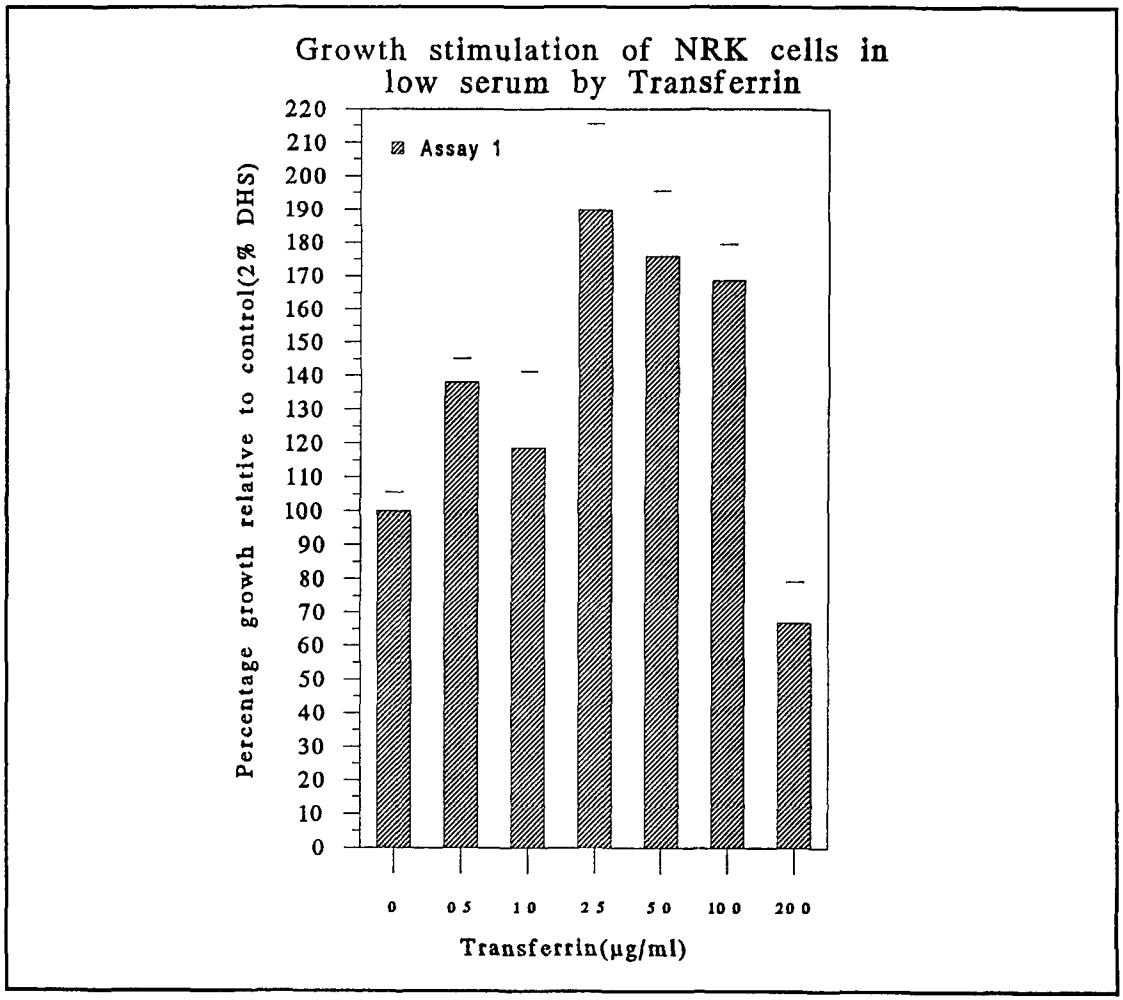


Figure 3.1.1.3 shows the growth response of NRK cells to bovine transferrin in low serum medium The results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as the end point and the results for 3 separate experiments are shown in Table 3 1 1 3

Table 3.1.1.3 Growth stimulation of transferrin (µg/ml)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
2% DHS	100 0 ± 5 49	100 0 ± 16 5	100 0 ± 9 0
+ 0 5 µg/ml	138 1 ± 7 08	134 2 ± 7 33	———
+ 1 0 µg/ml	118 6 ± 22 6	179 5 ± 36 9	186 5 ± 8 66
+ 2 5 µg/ml	189 7 ± 25 9	178 0 ± 47 6	135 0 ± 24 9
+ 5 0 µg/ml	175 8 ± 19 6	213 4 ± 29 6	163 0 ± 12 1
+ 10 0 µg/ml	168 6 ± 10 8	213 0 ± 31 8	156 0 ± 33 6
+ 20 0 µg/ml	66 95 ± 12 3	101 5 ± 13 7	140 7 ± 9 60

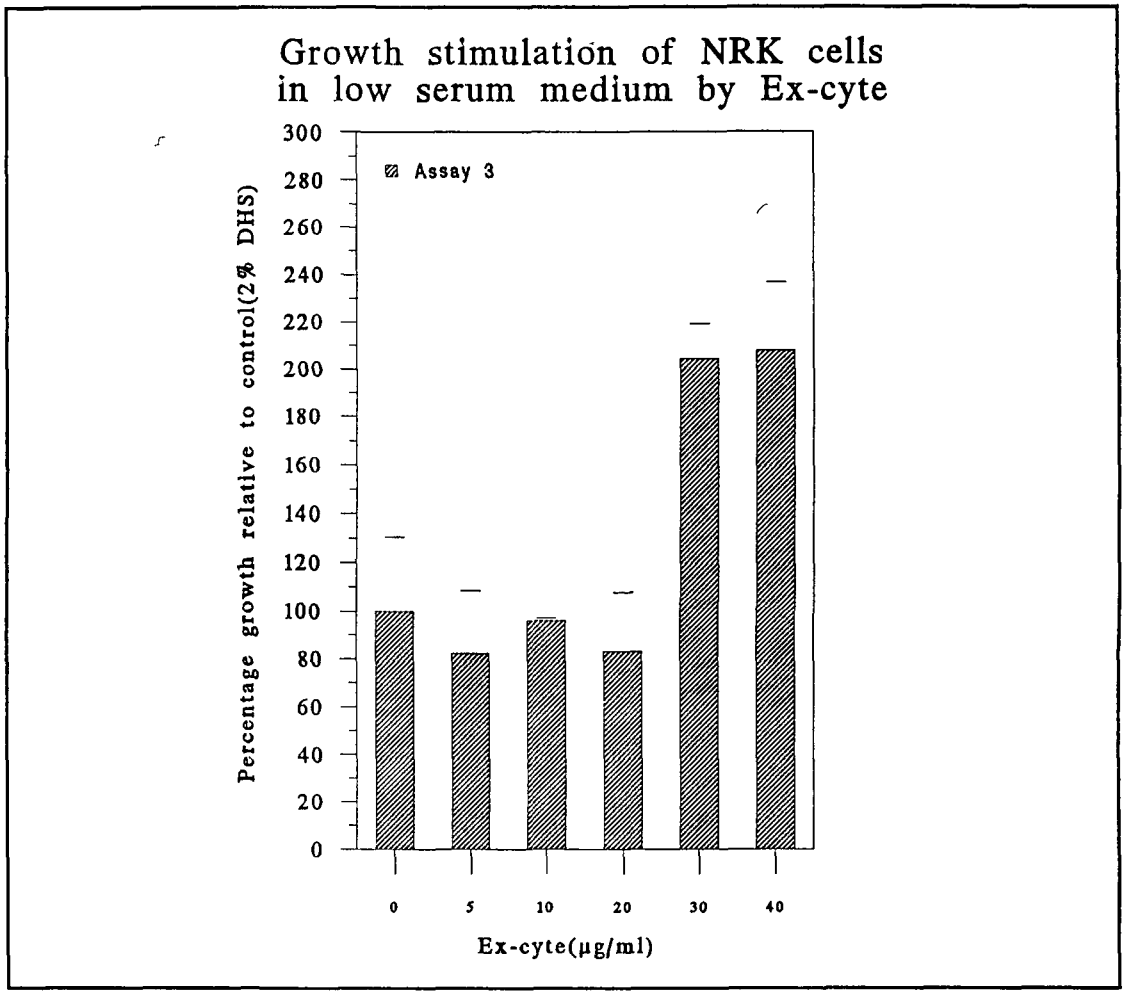


Figure 3.1.1.4 shows the growth response of NRK cells to Ex-cyte V in low serum medium The results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as the end point and the results for 3 separate experiments are shown in Table 3 1 1 4

Table 3.1.1.4 Growth stimulation of Ex-cyte (µg/ml)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
2% DHS	100 0 ± 30 0	100 0 ± 9 80	100 0 ± 30 3
+ 5 µg/ml	77 00 ± 26 0	135 0 ± 12 3	82 10 ± 26 2
+ 10 µg/ml	77 10 ± 5 85	129 0 ± 10 4	96 08 ± 0 98
+ 20 µg/ml	101 7 ± 14 9	83 10 ± 2 13	82 87 ± 24 6
+ 30 µg/ml	275 0 ± 24 8	160 1 ± 30 1	204 2 ± 14 8
+ 40 µg/ml	178 7 ± 28 7	159 8 ± 6 89	208 0 ± 28 7

3.1.2 EFFECT OF COMBINED FACTORS

From the results in section 3 1 1, each factor was found to have varying stimulating activity In the next step, a combination of the submaximal concentrations of these factors were made up and the effect on growth stimulation was compared to 5% FCS

The mixture of components is henceforth referred to as 'BITE' It consists of 0 5mg/ml BSA, 5μg/ml insulin, 5μg/ml transferrin and 20μg/ml Ex-cyte The ability of BITE to improve the growth of cells in low serum-supplemented medium was looked at The results are shown Table 3 1 2 1

Table 3.1.2.1 Enhancement of DHS with BITE

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
5% FCS	2754 ± 180	3008 ± 447	10612 ± 915
5% DHS	1621 ± 108	1788 ± 0 83	4886 ± 53 7
2% DHS	100 0 ± 11 8	100 0 ± 15 9	100 0 ± 8 98
1% DHS	10 99 ± 13 2	45 37 ± 6 51	2 19 ± 0 62
0 5% DHS	0 000 ± 0 00	0 000 ± 0 00	0 000 ± 0 00
2% DHS + BITE	568 0 ± 9 64	695 1 ± 142	1233 ± 172
1% DHS + BITE	323 4 ± 43 0	324 2 ± 36 6	189 0 ± 7 26
0 5% DHS + BITE	46 09 ± 8 65	8 81 ± 1 18	15 55 ± 5 50

Results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as the end point for three separate experiments

The results showed that for 2 of the 3 experiments, the BITE combination stimulated growth of 5 7-fold to 6 9-fold over the control (2% DHS) Overall stimulation relative to the control was not as good as that seen with 5% FCS or with 5% DHS The extent of stimulation was much greater in the third assay This may have been due to the low overall growth seen on the plate (which would make all the other factors appear more stimulatory in relation to 2% DHS)

At lower serum concentrations, a greater stimulatory effect was seen when 1% and 0 5% serum were compared with and without BITE 1% DHS alone stimulated cell growth but the extent of stimulation was very variable, between 2 2 - 45% of the activity seen with the control (2% DHS) When BITE was added, 2 of the 3 assays showed a 3-fold stimulation over the control (2% DHS) (1 89-fold in the third) 0 5% DHS alone did not support any growth but

addition of BITE showed a small amount of growth The growth with BITE was variable between the 3 assays (8 8 - 46% of the activity seen with 2% DHS)

The extent of stimulation for the combined BITE seen in these assays was only the equivalent of that seen with insulin in section 3 1 1 However, no control of each factor alone was tested in this experiment So an experiment was carried out in which maximal and submaximal concentrations (determined in section 3 1 1) of each of the BITE components were combined and compared The experiment was constructed so that insulin (I) and transferrin (T) would be kept constant and BSA and Ex-cyte varied The components were labelled as follows B_1 = BSA at 0 5mg/ml, B_2 = BSA at 1mg/ml, I_1 = insulin at 5μg/ml, I_2 = insulin at 10μg/ml, T_1 = transferrin at 5μg/ml, T_2 = transferrin at 10μg/ml, E_1 = Ex-cyte at 20μg/ml, E_2 = Ex-cyte at 30μg/ml This experiment was only carried out once with image analysis as the end point for experiments

Table 3.1.2 2 Growth response of the mixed BITE components

VARIABLE	I_1T_1	I_1T_2	I_2T_1	I_2T_2
Con	100 0 ± 34 0	100 0 ± 29 0	100 0 ± 15 0	100 0 ± 76 0
IT	811 0 ± 11 0	334 0 ± 115	740 0 ± 30 0	1190 ± 310
B_1	3883 ± 379	307 0 ± 155	6470 ± 209	12850 ± 1120
B_2	4084 ± 49 0	866 0 ± 57 0	10320 ± 560	19490 ± 1290
B_1E_1	4994 ± 256	891 0 ± 44 0	12970 ± 1140	1688 0 ± 1910
B_2E_1	5520 ± 191	768 0 ± 116	10980 ± 500	19420 ± 1760
B_1E_2	5022 ± 683	605 0 ± 117	9490 ± 2660	17450 ± 950
B_2E_2	5368 ± 238	782 0 ± 39 0	11520 ± 1370	19600 ± 2780

Results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=3) Image analysis was used as an end point Abbreviations Con = 2% DHS control, no BITEs components added, IT refers to insulin and transferrin combinations without BSA or Ex-cyte

Although the maximum stimulation was obtained at higher concentrations, the lower concentrations were optimum for cost/effectiveness reasons *i e* I_2T_2 was slightly better than I_1T_1, but was not twice as good Similarly for most ITs', B_2 was better than B_1, but doubling the concentration of BSA did not double the level of stimulation Increasing the concentration of Ex-cyte produced variable results depending on the concentration of the other components From the results, the BITE combination was chosen as 0 5mg/ml BSA, 5μg/ml insulin, 5μg/ml transferrin and 20μg/ml Ex-cyte (same combination of concentrations used in section 3 1 2 1)

3.1.3 UPGRADING OF DHS AND BITE

In this experiment a variety of growth factors and hormones were assayed for their growth stimulating ability on NRK cells using a 2% DHS background Due to the high number of variables, all experiments were carried out in 96-well plates and acid phosphatase and coulter counts were used as the end points (see section 2 9) Acid phosphatase has been shown to be a suitable end point for NRK cells (Martin and Clynes 1991) The results are shown in Tables 3 1 3 1 to 3

Table 3.1.3.1 Growth response of NRK cells to growth factors and hormones in low serum medium

VARIABLES	ASSAY 1		ASSAY 2		ASSAY 3	
	AP	CC	AP	CC	AP	CC
2% DHS + B	100 0 ± 5 00	100 0	100 0 ± 39 2	100 0	100 0 ± 11 9	100 0
+ 10μg/ml Dex	125 4 ± 14 5	61 00	54 77 ± 7 24	56 76	92 18 ± 1 98	28 60
+ 50μg/ml Dex	106 9 ± 8 16	64 27	45 12 ± 4 32	41 17	82 08 ± 0 99	23 60
+ 0 5U IL-1α	129 6 ± 16 6	53 59	72 46 ± 9 65	145 4	117 6 ± 1 98	48 80
+ 1 0U IL-1α	117 2 ± 8 16	63 83	83 42 ± 14 4	142 7	105 4 ± 1 98	56 74
+ 0 5U IL-1ß	174 6 ± 8 97	66 77	63 72 ± 11 0	173 3	147 9 ± 1 98	77 35
+ 1 0U IL-1ß	164 6 ± 17 5	85 18	106 3 ± 19 2	165 2	142 0 ± 1 98	66 42
+ 10ng/ml EGF + 1ng/ml α-FGF + Hep	288 2 ± 22 9	278 7	114 2 ± 13 1	236 9	125 6 ± 1 98	106 0
+ 10ng/ml EGF + 10ng/ml Dex	337 5 ± 20 8	126 4	177 9 ± 21 8	152 5	220 0 ± 32 9	80 28
+ 10ng/ml EGF + 1ng/ml ß-FGF	228 7 ± 10 6	255 4	93 97 ± 6 23	289 8	111 9 ± 17 1	69 12
+ 10ng/ml EGF + 10nM ßEs	256 2 ± 40 2	267 6	127 4 ± 33 5	252 3	136 6 ± 21 9	155 3

Results for acid phosphatase experiments are expressed as the average percentage growth relative to control (2% DHS + BITE) ± standard deviation (n=8) Only one reading was obtained when coulter counts were used as the end point Results for these are expressed as the percentage growth relative to 2% DHS + BITE Abbreviations EGF = Epidermal growth factor, ßEs = Estradiol, FGF = Fibroblastic growth factor, Hep = Heparin, B = BITE combination, IL = Interleukin, Dex = Dexamethazone

The combination of 10ng/ml EGF, 10ng/ml Dexamethazone and BITE showed the best growth with acid phosphatase as the end point Coulter count (CC) results showed the two combinations of EGF, ß-FGF and BITE or EGF, α-FGF, Heparin and BITE to be the best Dexamethazone alone showed variable inhibition in all assays with CC as the end point and in two of three assays with AP as the end point

Interleukin-1ß showed 40 - 74% stimulation above the control for 2 of 3 assays with AP as the end point Using CC as the end point, 15 - 33% inhibition was seen in 2 of 3 assays. IL-1α

showed variable results with both end points ß-Estradiol showed little or no stimulation alone Both α and ß-FGF with heparin showed stimulation α-FGF at the higher concentration (10ng/ml) was more effective than at 1ng/ml the extent of stimulation was about 20% with AP and between 1 56-fold and 2 40-fold stimulation with CC

Table 3.1.3.2 Growth response of NRK cells to growth factors and hormones in low serum medium

VARIABLES	ASSAY 1		ASSAY 2		ASSAY 3	
	AP	CC	AP	CC	AP	CC
2% DHS + B	100 0 ± 13 1	100 0	100 0 ± 18 8	100 0	100 0 ± 7 64	100 0
+ 1ng/ml α-FGF + Hep	89 32 ± 6 79	66 0	93 58 ± 3 90	82 30	91 51 ± 3 77	123 0
+ 10ng/ml α-FGF + Hep	122 2 ± 5 82	241 8	117 4 ± 5 50	160 5	120 7 ± 6 41	156 0
+ 1ng/ml ß-FGF + Hep	115 5 ± 6 79	79 30	121 1 ± 6 97	135 4	112 3 ± 4 43	139 0
+ 10ng/ml ß-FGF + Hep	116 5 ± 7 77	84 90	154 1 ± 15 6	144 2	120 7 ± 8 30	159 5
+ 10nM ßEs	103 0 ± 6 79	79 44	109 2 ± 10 1	125 2	98 11 ± 10 4	138 8
+ 100nM ßEs	107 8 ± 7 77	86 53	103 7 ± 11 9	118 4	96 23 ± 9 06	106 1

Results for acid phosphatase experiments are expressed as the average percentage growth relative to control (2% DHS + BITE) ± standard deviation (n=8) Results for coulter counts are expressed as the percentage growth relative to 2% DHS + BITE Abbreviations ßEs = Estradiol, FGF = Fibroblastic growth factor, Hep = Heparin, B = BITE combination

According to coulter counts and acid phosphatase, the best growth in this part, was achieved with the FGFs and BITE EGF was tested on its own as was IGF-I Neither showed as much stimulation as that seen with the combination of EGF and FGF or EGF and ß-Estradiol

Table 3.1 3 3 Growth response of NRK cells to growth factors in low serum medium

VARIABLES	ASSAY 1		ASSAY 2		ASSAY 3	
	AP	CC	AP	CC	AP	CC
2% DHS + B	100 0 ± 7 80	100 0	100 0 ± 5 49	100 0	100 0 ± 3 51	100 0
+ 10ng/ml IGF-I	98 09 ± 4 18	100 1	96 55 ± 6 89	101 0	93 85 ± 3 95	111 5
+ 50ng/ml IGF-I	96 96 ± 5 32	101 1	96 06 ± 4 33	122 7	91 79 ± 0 56	117 8
+ 10ng/ml EGF	92 39 ± 2 09	154 3	95 50 ± 5 91	194 9	104 6 ± 7 43	252 1
+ 50ng/ml EGF	91 63 ± 4 68	169 3	99 30 ± 6 30	156 4	103 1 ± 3 69	249 3
+ 10ng/ml EGF + IGF-I	131 5 ± 10 4	180 5	98 50 ± 3 94	213 2	114 3 ± 8 05	118 3

Results for acid phosphatase experiments are expressed as the average percentage growth relative to control (2% DHS + BITE) ± standard deviation (n=8) Results for coulter count experiments are expressed as the percentage growth relative to 2% DHS + BITE Abbreviations EGF = Epidermal growth factor, IGF = Insulin-like growth factor, B = BITE combination

The components which showed repeatedly good activity for both end points were ß-Estradiol and EGF, EGF, α-FGF and heparin, and EGF and Dexamethazone Surprisingly, when EGF was tested alone, no growth stimulation was seen with AP as the end point When CC was used as the end point, consistent stimulation was seen Overall, the most stimulatory combinations were EGF and Dexamethazone and EGF and 10nM ß-Estradiol

From the results obtained in section 3 1 3 1, it was decided to have a closer look at EGF, as EGF alone in the previous experiment showed good stimulation according to the cell counts but not with AP Triplicate repeats of each assay were set up in 96-well plates using AP activity as the end point A serial dilution of EGF was tested with and without BITE addition The level of serum was reduced to 1% DHS due to the high level of stimulation seen in section 3 1 3

EGF showed significant activity (Figure 3 1 3 5) EGF is a powerful mitogen and has been shown to elicit stimulatory and inhibitory effects on various cell types NRK cells showed significant stimulation with EGF on its own with best growth at 1-10ng/ml When BITE and EGF were combined, synergism occurred and the growth achieved was greater than that of 5% FCS

The work described above resulted in the development of a low serum medium (1% DHS) supplemented with BITE and EGF which stimulates growth as good as that achieved by 5% FCS

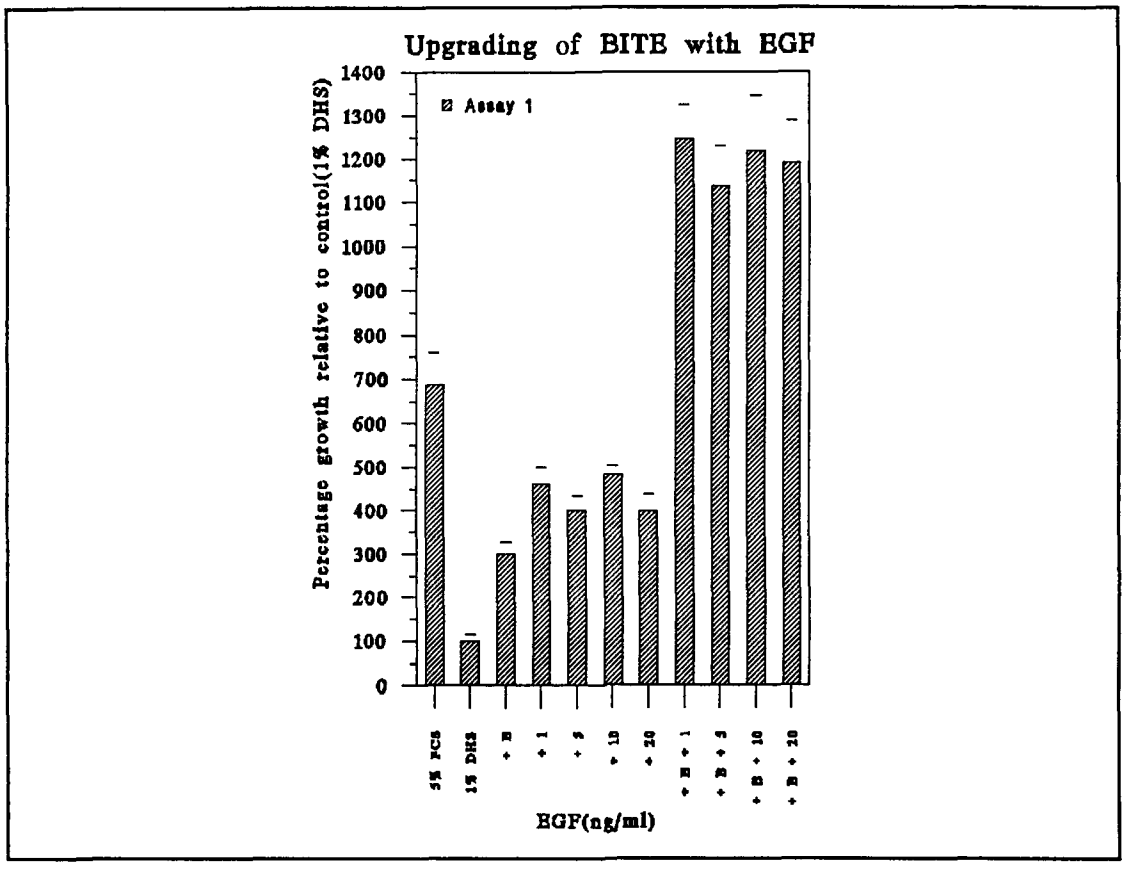


Figure 3.1.3.4 shows the effect of EGF with and without BITE for NRK cells Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations Con = Control (1% DHS), B = BITE combination Acid phosphatase was used as the end point for experiments and results for three separate experiments are shown in Table 3 1 3 4

Table 3.1.3.4 Combination of EGF and BITE

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
5% FCS	688 5 ± 72 0	868 0 ± 74 5	648 0 ± 63 9
1% DHS	100 0 ± 14 7	100 0 ± 8 86	100 0 ± 8 63
+ BITE	300 0 ± 26 8	353 0 ± 36 6	276 0 ± 16 8
+ 1ng/ml EGF	460 6 ± 38 7	529 7 ± 31 1	396 0 ± 33 9
+ 5ng/ml EGF	401 4 ± 33 8	505 8 ± 33 7	427 0 ± 28 9
+ 10ng/ml EGF	480 9 ± 23 1	475 0 ± 36 5	413 0 ± 17 5
+ 20ng/ml EGF	398 0 ± 37 5	477 7 ± 31 0	402 0 ± 27 8
+ B + 1ng/ml EGF	1246 ± 79 8	1541 ± 115	1070 ± 64 9
+ B + 5ng/ml EGF	1136 ± 93 1	1498 ± 143	1075 ± 80 4
+ B + 10ng/ml EGF	1216 ± 129	1266 ± 118	961 8 ± 74 2
+ B + 20ng/ml EGF	1189 ± 100	1287 ± 119	998 4 ± 106

3.1.4 EFFECT OF BASAL MEDIA ON GROWTH OF NRK CELLS

At the same time that the effects of various growth factors were investigated, it was decided to have a look at other basal media Up to now the basal medium used for the NRK cells was ATCC (1 1 ratio of DME and Ham's F12) The following basal media were investigated for NRK cells EBS = Earls Balanced Salts, PBS = Phosphate Buffered Saline, BME = Basal Medium Eagle, L-15 = Leibovitzs Medium, RPMI = RPMI-1640, GME = Glasgows Minimal Essential Medium, MEM = Minimal Essential Medium, McCoys 5a, Ham's F12, ATCC The contents of each basal medium is shown in Appendix A

Experiments were carried out in triplicate on 24-well plates Data were obtained by image analysis 2% DHS was the basal serum level with assays carried out both in the presence and absence of BITE As NRK cells are regularly maintained in DME, this was chosen as the basal medium with which to compare the other basal media The results are shown in Figure 3 1 4

McCoys 5a was the only basal medium to show better growth (3 77-fold) than that obtained by DME alone With BITE addition, the best growth in the experiment was seen with 18 8-fold stimulation over DME alone and 1 88-fold stimulation over DME + BITE After McCoys 5a, GME showed the best growth with BITE (9-fold increase on DME alone), but alone showed only 1 6% of the growth achieved by DME alone

EBS and PBS with and without BITE addition did not support cell growth of NRK cells Microscopic examination before staining showed no appreciable attachment of cells in PBS $\pm$ BITE, while with EBS as the basal medium, some attachment was seen BME showed slightly better growth and addition of BITE improved growth RPMI-1640 supported only 0 4% of the growth in DME alone in the absence of BITE, and 72 8% in its presence Alone, MEM was not capable of supporting growth but when BITE was added the growth was 2 5-fold that obtained by DME alone (only 25% of that achieved by DME + BITE)

Leibovitz-15 could not support growth alone and the addition of BITE only resulted in 65% of the growth achieved by DME alone Ham's F12 alone supported little growth (30% of that supported by DME) and addition of BITE only showed the same stimulation as that achieved by DME alone ATCC, the basal medium for many serum-free formulations, did not support much growth These result show that of all the basal media tested, that McCoys 5a either with or without BITE addition was the most stimulatory

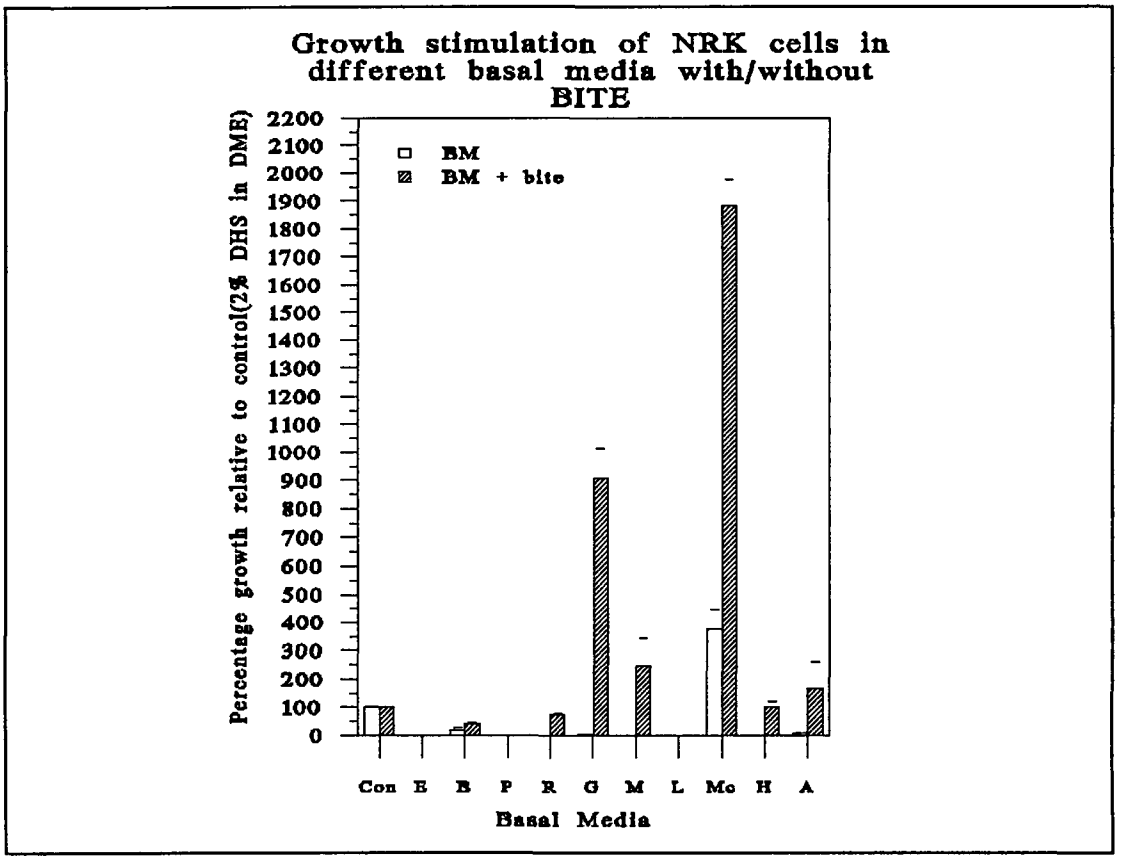


Figure 3.1.4 shows the effect of different basal media both in the presence and absence of BITE for NRK cells Results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8) Abbreviations Con = Control medium (DME), E = EBS, B = BME, L = Leibovitz 15, P = PBS A, R = RPMI-1640, G = GME, M = MEM, Mc = McCoys 5a, H = Ham's F12, A = ATCC, b = BITE (see section 3 1 2)

Table 3.1.4 Effect of different basal media

VARIABLES	ASSAY 1	VARIABLES	ASSAY 1
2% DHS	100 0 ± 4 20	2% DHS + b	1003 0 ± 193
E	0 000 ± 0 00	M	0 000 ± 0 00
E + b	0 000 ± 0 00	M+ b	246 9 ± 98 3
B	19 60 ± 9 00	L	0 000 ± 0 00
B + b	39 81 ± 8 43	L + b	0 65 ± 0 30
P	0 000 ± 0 00	Mc	377 7 ± 68 0
P + b	0 000 ± 0 00	Mc + b	1884 ± 94 0
R	0 400 ± 0 20	H	0 350 ± 0 23
R + b	72 80 ± 5 70	H + b	101 2 ± 19 0
G	1 620 ± 0 16	A	7 010 ± 0 40
G + b	907 0 ± 105	A + b	165 3 ± 95 0

3.1.5 FURTHER COMPARISON OF ATCC AND McCOYS 5a

From the results obtained in section 3 1 3, the combination EGF and BITE showed better growth than 5% FCS In section 3 1 4, it was found that McCoys 5a as the basal medium supported better growth of NRK cells than ATCC either with or without BITE
It was decided to remove serum altogether and study the combined effects of EGF and McCoys 5a medium As Ex-cyte V was known to contain BSA fraction V and since BSA was already in the BITE combination, it was decided to try a different Ex-cyte Ex-cyte III, which contained the same lipoprotein mixture but no BSA This was not found to be stimulatory (Appendix B) and as a result was subsequently left out With the removal of serum, the concentrations of BSA and insulin were increased

The ability of the two basal media, ATCC and McCoys 5a, to support growth under serum limiting conditions were investigated Both basal media were examined in the presence of mixtures of insulin (10μg/ml), transferrin (5μg/ml), EGF (10ng/ml), BSA (1mg/ml) and laminin (100μg/ml) This is BITE plus EGF, plus laminin and minus Ex-cyte The laminin was incorporated into the assay to see if attachment factors were necessary for growth in totally serum-free conditions Laminin can be added to the plate and allowed to dry before adding the cells or it can be incorporated into the growth medium In this assay, laminm was included in the medium
In preliminary experiments, it had been found that NRK cells did not form a large pellet when trypsin inhibitor was used to stop the action of trypsin It may have been that trypsin-trypsin inhibitor complexes impeded the settling of cells It was also possible that the trypsin inhibitor was not fully successful in deactivating the trypsin, resulting in some continued proteolytic activity on the cells To overcome this problem, 5% DHS was used to stop the action of trypsin After centrifuging, all the supernatant was removed, the cells were resuspended in 10ml basal medium (without serum) The cells were centrifuged again, the supernatant decanted and the cells were resuspended in basal medium without serum In subsequent assays using trypsin inhibitor, it was found that by increasing the centrifugal speed to 1,500rpm it was possible to get a good pellet (comparable to that obtained when serum was used to inactivate trypsin)

The results are shown in Figure 3 1 5 1 Using ATCC as the basal medium, insulin alone or the combination of insulin, transferrin and EGF were found to be most stimulatory, either with

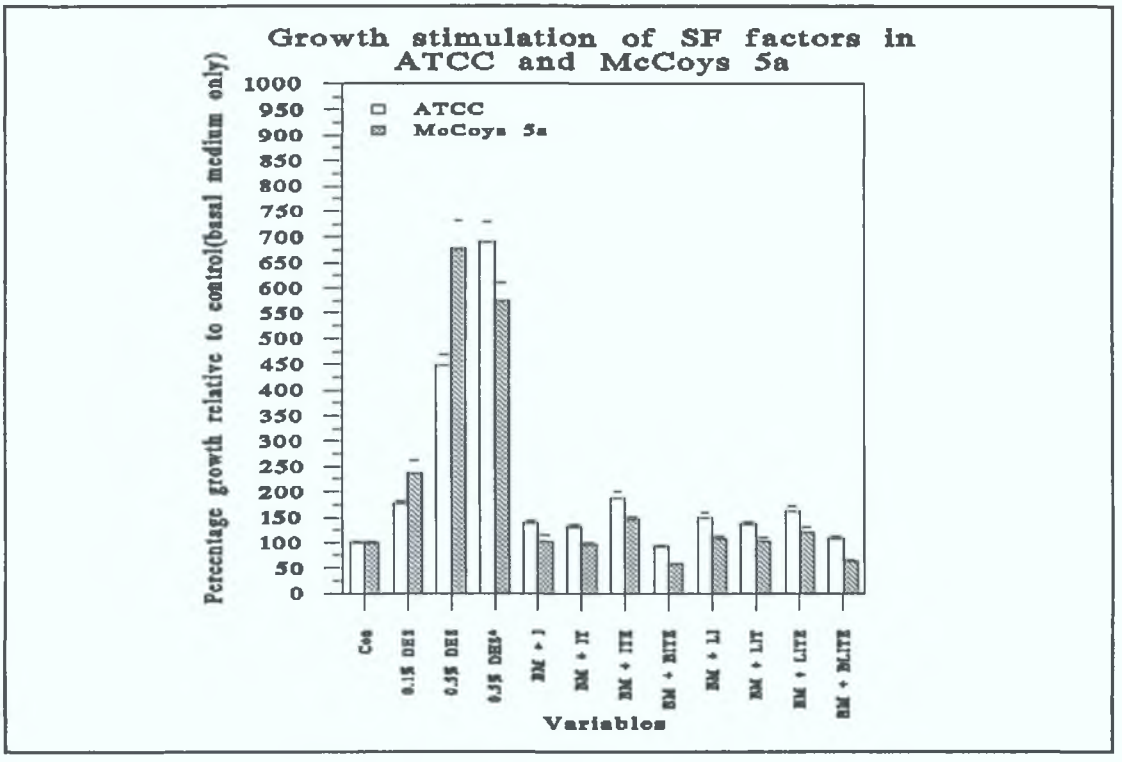


Figure 3.1.5.1 shows the effect of ATCC and McCoys 5a as the basal media with SF factors for NRK cells. Results are expressed as the average percentage growth relative to control (respective basal media) $\pm$ standard deviation (n=8). Abbreviations: Con = Control medium (ATCC or McCoys alone); I = 10μg/ml insulin; B = 1mg/ml BSA; L = 1μg/ml laminin; T = 5μg/ml transferrin; BM = basal medium. Acid phosphatase was used as the end point for experiments and results for two separate assays are shown in Table 3.1.5.1.

Table 3.1.5.1 Effect of different basal media

BM	ATCC		McCOYS 5a	
VARIABLES	ASSAY 1	ASSAY 2	ASSAY 1	ASSAY 2
Con	100.0 $\pm$ 6.93	100.0 $\pm$ 5.08	100.0 $\pm$ 4.07	100.0 $\pm$ 3.00
0.1% DHS	199.0 $\pm$ 2.83	176.6 $\pm$ 8.12	266.8 $\pm$ 16.8	236.5 $\pm$ 24.9
0.5% DHS	453.0 $\pm$ 90.7	448.6 $\pm$ 7.61	760.2 $\pm$ 53.9	678.5 $\pm$ 53.9
0.5% DHS*	659.8 $\pm$ 7.35	689.7 $\pm$ 9.14	581.2 $\pm$ 23.4	573.7 $\pm$ 36.2
BM + I	150.0 $\pm$ 9.70	137.4 $\pm$ 7.61	107.5 $\pm$ 4.60	103.0 $\pm$ 12.6
BM + IT	139.2 $\pm$ 5.98	130.8 $\pm$ 10.7	105.5 $\pm$ 6.66	96.73 $\pm$ 3.88
BM + ITE	198.0 $\pm$ 9.80	187.8 $\pm$ 8.63	173.8 $\pm$ 8.60	145.1 $\pm$ 6.31
BM + BITE	82.90 $\pm$ 4.31	90.60 $\pm$ 4.67	66.24 $\pm$ 4.42	57.48 $\pm$ 2.10
BM + LI	145.7 $\pm$ 11.8	147.7 $\pm$ 10.3	134.3 $\pm$ 9.508	106.5 $\pm$ 5.95
BM + LIT	131.4 $\pm$ 3.92	135.5 $\pm$ 6.54	102.8 $\pm$ 8.33	101.5 $\pm$ 8.16
BM + LITE	154.9 $\pm$ 10.8	160.7 $\pm$ 10.3	135.0 $\pm$ 6.16	119.2 $\pm$ 11.2
BM + BLITE	90.68 $\pm$ 3.92	106.2 $\pm$ 6.54	79.08 $\pm$ 4.50	63.78 $\pm$ 3.05

or without laminin Addition of transferrin appeared to slightly inhibit the action of insulin When EGF was added, stimulation was increased again (85 - 87 8% above the control (ATCC, no serum) Incorporation of BSA resulted in total loss of stimulation and was slightly inhibitory The incorporation of laminin into these combinations did not affect growth (when the standard deviations were taken into account) except for the combination of insulin, transferrin and EGF, where there was a slight loss in stimulation

When McCoys 5a was used as the basal medium, insulin alone or with transferrin did not cause any stimulation When EGF was added to insulin and transferrin, some variable stimulation occurred (45 1 - 73 8% above the control) The combination BITE was 34 - 40% inhibitory Laminin, when added to the combinations, did not improve growth As seen with ATCC, BSA resulted in a loss of stimulation with the 'ITE' combination This was the first time that BSA and transferrin were found to be inhibitory at these concentrations This inhibitory response may have been due to the total absence of serum or the basal medium

To compare the ability of the two basal media to support growth, a fixed control on every plate was required (see 0 5% DHS*, Table 3 1 5 1) On the plates where ATCC was being used, 0 5% DHS in McCoys was added Therefore, when the cell suspension was added, the cells were in a 50 50 ratio of ATCC McCoys medium A similar control containing 0 5% DHS in ATCC was used when McCoys 5a was investigated When the growth in 0 5% DHS was then expressed as a ratio over the growth in this 0 5% DHS* control, the results showed that for ATCC the ratio was 0 67, obtained as the average from two assays For McCoys 5a, the ratio was 1 24 Clearly McCoys 5a was almost twice as good as ATCC for supporting cell growth in very low serum

3.1.6 COMPARISON WITH COMPONENTS FROM OTHER SFM

As MDCK and CHOK1 cells grew successfully in the serum-free media cited for each, it was decided to try the components used in these SF media on NRK cells under serum-free conditions From previous experiments (sections 3 1 4 - 3 1 5), McCoys 5a medium was found to support growth better than ATCC in the presence of very low serum concentrations Therefore, each set of variables was tested with three different basal media *i e* the basal media for the two SFM (ATCC and Ham's F12) and McCoys 5a In addition, EGF which had been found to be very stimulatory for NRK cells (Figure 3 1 3 2), was added in to the SF media This experiment was only carried out once Trypsin inhibitor was added to the serum-free cell stocks to protect the cells from the possible proteolytic action of trypsin on cells under serum-free conditions The results are shown in Tables 3 1 6 1 and 2

Looking at the 1% DHS controls for the different basal medium, both Ham's F12 and ATCC show a 2-fold to 3-fold increase in growth over the SFM, while for McCoys 5a there was a 7-fold to 8-fold increase (Tables 3 1 6 1 and 2) In section 3 1 4, McCoys 5a was shown to be twice as good as ATCC These results could indicate that there may be some factor affecting cell growth adversely in ATCC or Ham's F12, that the addition of serum did not totally overcome McCoys 5a may also have some factor(s) not present in the other basal media which is(are) important for the growth and maintenance of NRK cells

Table 3.1 6.1 Growth response of NRK cells to components in MDCK SFM

VARIABLES	ATCC	Ham's F12	McCoys 5a
1% DHS	257 0 ± 12 0	276 2 ± 11 9	882 6 ± 34 8
Basal medium	112 0 ± 2 00	113 9 ± 1 98	84 06 ± 4 35
SFM	100 0 ± 2 00	100 0 ± 0 99	100 0 ± 2 89
- 5μg/ml Insulin	104 0 ± 3 00	108 9 ± 1 98	78 26 ± 3 81
- 5μg/ml Transferrin	97 00 ± 2 00	98 02 ± 1 98	101 4 ± 2 89
- 25ng/ml PGE_1	97 00 ± 3 00	98 02 ± 1 98	96 38 ± 6 52
- 50nM Hydrocortisone	96 00 ± 3 00	97 03 ± 1 98	99 27 ± 5 07
- 5pM Triiodothronine	95 00 ± 4 00	95 05 ± 1 98	94 20 ± 7 25
+ 10ng/ml EGF	113 0 ± 4 00	108 9 ± 2 97	123 9 ± 3 62

Results are expressed as the average percentage growth relative to control (SFM as described by Taub *et al* (1979) with three different basal media ATCC, Ham's F12 and McCoys 5a) ± standard deviation (n=8) Acid phosphatase was used as the end point for assays Abbreviations PGE_1 = Prostaglandin E_1

The growth response of NRK cells to the components used in the SFM designed for MDCK cells are shown in Table 3 1 6 1 When the complete SFM was compared to the basal medium, the basal medium was slightly better than the SFM in both cases EGF addition in all cases stimulated growth, however the stimulation was very low, reaching a maximum of 24% growth over the control (for McCoys 5a) Removal of any of the factors individually did not result in a loss of activity, except for insulin when McCoys was used as the basal medium

Table 3 1 6 2 shows the results when the components from the SFM designed for CHOK1 cells were tested on NRK cells in SFM ATCC and Ham's F12 were better alone than with the SF components For McCoys 5a, the SFM was better than the basal medium alone Only on removal of insulin or Fe_2SO_4 from the complete SFM, was loss of stimulation seen EGF had no effect on addition to the CHOK1 SFM

Table 3.1.6.2 Growth response of NRK cells to components in CHOK1 SFM

VARIABLES	ATCC	Ham's F12	McCoys 5a
1% DHS	197 2 ± 10 4	266 9 ± 10 4	760 6 ± 47 5
Basal medium	117 9 ± 1 89	113 2 ± 2 83	68 75 ± 2 50
SFM	100 0 ± 2 83	100 0 ± 2 68	100 0 ± 8 12
- 10μg/ml Insulin	111 3 ± 1 89	105 7 ± 3 77	73 75 ± 3 12
- 5μg/ml Transferrin	99 06 ± 2 83	95 28 ± 1 89	93 12 ± 3 75
- 10nM NEAA	96 23 ± 2 83	94 34 ± 2 83	93 75 ± 3 12
- 0 3μM Linoleic Acid	100 0 ± 2 83	97 20 ± 1 89	97 50 ± 2 50
- 60nM $CaCl_2$	100 0 ± 1 89	94 30 ± 6 60	100 0 ± 5 0
- 5μM Fe_2SO_4	98 11 ± 1 89	95 70 ± 2 83	76 25 ± 2 50
+ 10ng/ml EGF	100 9 ± 1 89	105 6 ± 2 83	96 87 ± 4 40

Results are expressed as the average percentage growth relative to control (SFM as described by Mendiaz with three different basal media ATCC, Ham's F12 and McCoys 5a) ± standard deviation (n=8) Acid phosphatase was used as the end point for assays

The SFM described for the growth of MDCK and CHOK1 cells were unable to support the growth of NRK cells Again, McCoys 5a basal medium appeared to be the best These results suggested the combination of insulin, Fe_2SO_4 and EGF with McCoys 5a should be used as a basis to develop a SFM for NRK cells Note In a thymidine incorporation assay (section 3 5 4 13), cells which were inoculated in ATCC, had rounded up and appeared to be peeling off the plate, after 24 hours When this assay was repeated using McCoys 5a as the basal medium, cells did not peel off the plate This indicated how critical McCoys 5a was for sustaining the viability of NRK cells under serum-free conditions

3.1.7.1 EFFECTS OF SELECTED VARIABLES ON NRK CELLS IN SFM

Following from section 3 1 5, McCoys 5a was used as the basal medium supplemented with insulin, Fe_2SO_4 and EGF The addition of trypsin inhibitor and EDTA were investigated Trypsin inhibitor was added to the SF stocks of cells in section 3 1 5 in order to protect the cells from the effect of residual trypsin No control had been included to see if trypsin inhibitor had an adverse effect EDTA was included as it has been reported (Bertheussen, 1993) to act as a chelator of toxic factors The experiment was set up using all these factors in the serum-free medium Each factor was left out individually to see what the impact on cell growth would be The results are shown in Figure 3 1 7 1

As seen before, the complete serum-free medium was only 10% better than the basal medium alone This appeared disappointing until the effect of the omission of EGF was noted When EGF was left out, there was a significant increase in growth (even though the standard deviations were high) When insulin or Fe_2SO_4 were left out individually, there was a 25 - 30% and 15 - 25% reduction in growth respectively The incorporation of trypsin inhibitor appeared to have no effect in 2 of the 3 assays, with only a small increase in the third assay Thus, it was not necessary for cell growth/maintenance in SFM Omission of EDTA showed statistically insignificant differences (given errors on the control as well as on the 'EDTA' results The effect of EDTA would have to be re-evaluated

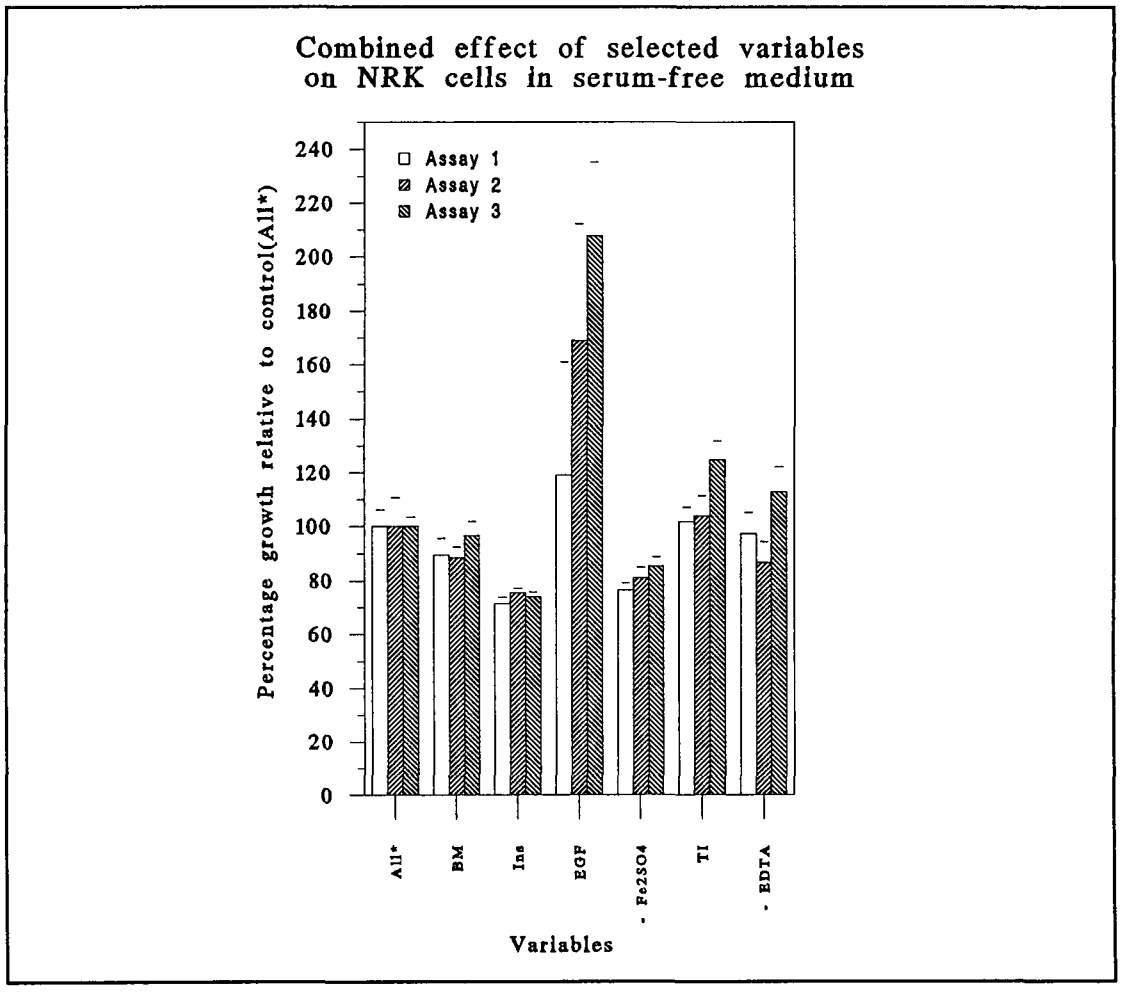


Figure 3.1.7.1 shows the growth response of NRK cells to variables found to be stimulatory under serum-free conditions in section 3 1 6 The results are expressed as the average percentage growth relative to control (where all variables are included) ± standard deviation (n=8) The basal medium was McCoys 5a All* / All components = 10μg/ml insulin, 5ng/ml EGF, 1 39μg/ml Fe_2SO_4, 10μg/ml trypsin inhibitor and 5μg/ml EDTA Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 1 7 1

Table 3.1.7.1 Effect of selected variables on the growth of NRK cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	1716 ± 70 4	1761 ± 112	1971 ± 107
Basal medium	89 56 ± 6 09	88 69 ± 3 77	96 55 ± 5 17
All components	100 0 ± 6 09	100 0 ± 10 8	100 0 ± 3 30
- Insulin	71 30 ± 2 61	75 47 ± 1 89	74 14 ± 1 72
- EGF	199 1 ± 41 7	168 9 ± 44 4	207 8 ± 27 6
- FE2SO4	76 52 ± 2 61	81 13 ± 3 77	85 34 ± 3 45
- TI	101 7 ± 5 22	103 8 ± 7 55	125 0 ± 6 89
- EDTA	97 40 ± 7 83	86 79 ± 7 55	112 9 ± 9 48

3.1.7.2 Effect of Interleukins, trace elements and phosphatides

For these experiments, the SFM consisted of McCoys 5a as the basal medium supplemented with 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 5μg/ml EDTA, 1x NEAA and 10nM Na_2SeO_3 Tables 3 1 7 2 1 to 3 show the results obtained when the SFM was supplemented with Interleukins 1α and 6, lysophosphatidic and phosphatidic acid and the trace elements used by Mendiaz *et al* (1986) Trace elements and their concentrations are shown in Appendix J

The results for the interleukins showed that IL-6 was stimulatory although the extent of stimulation varied between the two experiments IL-1α had little or no effect in one experiment and was inhibitory in the second

Table 3.1.7.2.1 Growth stimulatory effects of IL-1α and IL-6 on NRK cells

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	1975 $\pm$ 70 4	2122 $\pm$ 102
Basal medium	64 46 $\pm$ 6 09	88 69 $\pm$ 6 18
SFM*	100 0 $\pm$ 20 7	100 0 $\pm$ 10 2
+ 50 EU/ml IL-6	144 6 $\pm$ 19 5	146 7 $\pm$ 18 9
+ 100 EU/ml IL-6	110 1 $\pm$ 47 2	162 5 $\pm$ 20 5
+ 200 EU/ml IL-6	118 8 $\pm$ 15 8	193 8 $\pm$ 26 1
+ 0 1 μg/ml IL-1α	67 38 $\pm$ 19 2	121 4 $\pm$ 10 8
+ 1 0 μg/ml IL-1α	80 19 $\pm$ 11 5	133 2 $\pm$ 14 7
+ 10 0 μg/ml IL-1α	86 48 $\pm$ 12 5	122 3 $\pm$ 13 2

Results are expressed as the average percentage growth relative to control (SFM*) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point for 2 separate experiments The SFM* contained (in addition to McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 5μg/ml EDTA, 5μg/ml transferrin, 1X NEAA and 10nM Na_2SeO_3 Abbreviations IL-6 = Interleukin 6 (expressed as EU/ml), IL-1α = Interleukin 1α (expressed as μg/ml)

The trace elements were used at the concentrations employed by Mendiaz *et al* , (1986) Results showed the trace elements to be stimulatory although the standard deviations were high (Table 3 1 7 2 2) Stimulation was good with MoO_7, $CuSO_4$, $SnCl_2$ and $NiCl_2$ The best growth was obtained with $ZnSO_4$ where stimulation of 60% above the control was achieved

Phosphatidic acid was slightly stimulatory reaching a maximum stimulation of 30% above the control at 5 - 10μg/ml (Table 3 1 7 2 3) Lysophosphatidic acid showed some stimulation, but when the standard deviations were taken into account, the effect was negligible

Table 3.1.7.2.2 Growth stimulatory effects of trace elements on NRK cells

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	2577 ± 70 4	3030 ± 242
Basal medium	61 82 ± 12 3	60 61 ± 9 10
SFM*	100 0 ± 10 0	100 0 ± 8 64
+ 1nM MoO_7	145 6 ± 30 2	148 5 ± 24 2
+ 1nM Mn_2SO_4	127 8 ± 24 1	121 0 ± 21 1
+ 1nM Cu_2SO_4	141 9 ± 21 9	140 9 ± 12 1
+ 50nM SiO_3	122 9 ± 30 9	106 1 ± 30 2
+ 0 5nM $SnCl_2$	151 5 ± 11 2	143 9 ± 21 2
+ 0 5nM $NiCl_2$	127 9 ± 29 5	159 1 ± 24 4
+ 50nM Zn_2SO_4	162 3 ± 12 6	163 6 ± 30 3

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 5μg/ml EDTA, 5μg/ml transferrin, 1X NEAA and 10nM Na_2SeO_3

Table 3.1.7.2.3 Growth stimulatory effects of lysophosphatidic and phosphatidic acid on NRK cells

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	1662 ± 70 4	1223 ± 50 8
Basal medium	56 66 ± 10 7	42 57 ± 3 24
SFM*	100 0 ± 10 4	100 0 ± 14 9
+ 5nM NH_4VO_3	154 4 ± 5 37	118 2 ± 7 43
+ 1μg/ml LPA	130 9 ± 21 1	102 7 ± 14 1
+ 5μg/ml LPA	88 15 ± 6 35	117 6 ± 10 8
+ 10μg/ml LPA	93 33 ± 14 8	104 6 ± 4 44
+ 1μg/ml PA	128 3 ± 17 6	115 1 ± 8 41
+ 5μg/ml PA	133 5 ± 15 9	115 5 ± 8 41
+ 10μg/ml PA	126 0 ± 8 52	130 0 ± 7 67

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition to McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 5μg/ml EDTA, 5μg/ml transferrin, 1X NEAA and 10nM Na_2SeO_3 Abbreviations PA = phosphatidic acid, LPA = lysophosphatidic acid

3.1.7.3 Effect of vitamins, BSA and growth factors on NRK cells in SFM

In these experiments, the basic SFM was changed again NEAA and Na_2SeO_3 were left out until the effect of these components on the NRK cells had been established The effects of two vitamin complexes were investigated In addition, the effect of BSA fraction V and the 0 5M NaCl fraction from the Heparin Sepharose fractionation of BSA (section 3 5 5) were investigated Growth factors EGF, ß-FGF and PDGF were also tested for growth stimulation The results are shown in Tables 3 1 7 3 1 to 3

The two vitamin complexes were BME-vitamins and MEM-vitamins (Table 3 1 7 3 1) These were supplied in 100X form and diluted down in basal medium and tested at 0 1, 0 5 and 1 0X concentrations The results showed both vitamin complexes to be inhibitory, with growth appearing only slightly better than the basal medium For MEM vitamins, increasing the concentration resulted in a small increase in inhibition, while for BME the trend was not so distinct McCoys 5a has a good selection of vitamins already and the addition of more vitamins may have resulted in the concentrations becoming too high and as a result inhibitory $SnCl_2$ was also included on this plate and appeared to be inhibitory (unlike in section 3 1 6, where 40 - 50% stimulation over the control was seen) Phosphatidic acid and IL-6 were found to be mostly inhibitory in both experiments Phosphatidic acid reached maximum stimulation at 5µg/ml Na_2SeO_3 was also inhibitory

Table 3.1.7.3.1 Growth stimulatory effects of vitamin complexes on NRK cells

VARIABLES	ASSAY 1	ASSAY 2	VARIABLES	ASSAY 1	ASSAY 2
1% DHS	1048 ± 64 3	710 4 ± 52 5	1% DHS	866 ± 68 0	1073 ± 48 7
Basal medium	38 93 ± 2 68	54 45 ± 5 40	Basal Medium	54 00 ± 4 00	36 50 ± 3 24
SFM*	100 0 ± 7 39	100 0 ± 13 4	SFM	100 0 ± 9 87	100 0 ± 6 56
+ 0 1 BME	71 43 ± 14 3	54 96 ± 5 56	+ 1µg/ml PA	68 66 ± 3 50	68 92 ± 8 11
+ 0 5 BME	62 50 ± 7 14	58 29 ± 4 07	+ 5µg/ml PA	108 3 ± 6 26	193 47 ± 11 3
+ 1 0 BME	65 48 ± 10 4	60 5 ± 4 51	+ 10µg/ml PA	62 57 ± 3 59	65 31 ± 4 16
+ 0 1 MEM	86 61 ± 6 26	72 57 ± 4 98	+ 50EU/ml IL-6	57 33 ± 5 16	61 00 ± 5 20
+ 0 5 MEM	72 02 ± 3 69	69 94 ± 10 6	+ 100EU/ml IL-6	60 66 ± 5 16	66 44 ± 8 73
+ 1 0 MEM	73 21 ± 6 08	63 30 ± 13 1	+ 200EU/ml IL-6	68 66 ± 8 64	65 31 ± 6 92
+ $SnCl_2$	89 54 ± 6 95	65 57 ± 7 69	+ Na_2SeO_3	64 00 ± 5 83	68 69 ± 3 01

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition to McCoys 5a) 10µg/ml insulin, 1 39µg/ml Fe_2SO_4 and 5µg/ml EDTA

Both BSA and the 0 5M NaCl fraction showed increasing inhibition with increasing concentrations The 0 5M NaCl fraction was less inhibitory at 0 1 and 0 5mg/ml than the BSA itself but at 1 0mg/ml equal inhibition was exhibited by both In this experiment, all trace elements were inhibitory

Table 3.1.7.3.2 Growth response of NRK cells BSA, 0 5M NaCl fraction and trace elements

VARIABLES	ASSAY 1	ASSAY 2	VARIABLES	ASSAY 1	ASSAY 2
1% DHS	1027 ± 76 7	914 7 ± 62 7	1% DHS	643 1 ± 41 2	1003 ± 43 8
Basal medium	66 85 ± 7 65	36 19 ± 4 18	Basal Medium	49 02 ± 3 92	38 36 ± 4 11
SFM*	100 0 ± 7 72	100 0 ± 9 50	SFM*	100 0 ± 8 12	100 0 ± 7 09
+ 0 1 BSA	35 77 ± 5 82	57 06 ± 10 4	+ MoO_7	62 74 ± 7 84	77 85 ± 9 06
+ 0 5 BSA	-6 39 ± 2 14	2 660 ± 1 69	+ $Si0_3$	53 27 ± 3 59	66 57 ± 3 15
+ 1 0 BSA	-10 2 ± 2 55	-0 44 ± 1 08	+ $SnCl_2$	41 45 ± 2 87	63 40 ± 7 74
+ 0 1 BSAf	55 18 ± 8 19	77 77 ± 7 43	+ NH_4VO_3	37 91 ± 5 03	61 37 ± 2 81
+ 0 5 BSAf	0 730 ± 4 59	12 00 ± 8 00	+ $MnSO_4$	36 97 ± 3 48	67 46 ± 7 53
+ 1 0 BSAf	-10 2 ± 2 55	1 330 ± 4 00	+ $CuSO_4$	39 49 ± 2 64	61 18 ± 2 24
+ All*	66 42 ± 5 11	58 00 ± 7 20	+ $NiCl_2$	35 95 ± 2 95	60 27 ± 12 3

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition to McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4 and 5μg/ml EDTA Abbreviations BSAf = 0 5M NaCl fraction from Heparin Sepharose experiments (see section 3 5 4), All* as before

Table 3.1.7.3.3 Growth stimulatory effects of growth factors on NRK cells

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	1115 ± 29 7	967 1 ± 64 5
Basal medium	62 02 ± 5 05	34 21 ± 1 31
SFM*	100 0 ± 9 02	100 0 ± 6 27
+ 1ng/ml EGF	91 28 ± 8 89	57 89 ± 7 06
+ 10ng/ml EGF	97 97 ± 10 3	69 36 ± 6 82
+ 1ng/ml ß-FGF	229 3 ± 21 9	167 3 ± 16 2
+ 10ng/ml ß-FGF	214 9 ± 11 3	143 2 ± 15 6
+ 50ng/ml ß-FGF	110 7 ± 12 1	66 58 ± 4 71
+ 1ng/ml PDGF	89 19 ± 9 84	68 20 ± 5 29
+ 10ng/ml PDGF	150 8 ± 8 37	148 5 ± 10 7

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition to McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4 and 5μg/ml EDTA

For the growth factors, EGF was found to be slightly inhibitory while ß-FGF and PDGF were found to be stimulatory. EGF was found to exhibit 10 - 40% inhibition in the concentration range 1 - 10ng/ml. ß-FGF was stimulatory at 1 - 10ng/ml with the best growth at 1ng/ml (1.67-fold to 2.30-fold over the control). At 10ng/ml, ß-FGF was only slightly less stimulatory, but at 50ng/ml, it had either lost its stimulatory ability or had become inhibitory. PDGF was tested at 1 - 10ng/ml. Best growth was observed at 10ng/ml with 50% stimulation above the control.

3.1.7.4 Effect of selected variables on the growth of NRK cells in SFM

With the fact that so many of the factors were inhibitory in these experiments and stimulatory in earlier experiments (section 3.1.7), it was necessary to re-examine some of the factors. It may be that in the presence of different components in the SFM or the different basal media, factors may exhibit stimulatory or inhibitory effects.

Na_2SeO_3 and NEAA showed the most consistent stimulation. The extent of stimulation was low, about 40% above the control. The trace elements were tested again. $ZnSO_4$, MoO_7, $SnCl_2$ and $CuSO_4$ showed stimulation in one of the assays and no effect in the other. The complete cocktail of trace elements (referred to as All*) was no more stimulatory than NEAA. In addition, IL-4 and serum-free Briclone were also tested. Briclone is a SF supplement produced in the NCTCC which is known to contain IL-6 (normally used at 5% (vol/vol) concentration).

Table 3.1.7.4.1 Growth response of NRK cells to selected variables

VARIABLES	ASSAY 1	ASSAY 2	VARIABLES	ASSAY 1	ASSAY 2
SFM*	100.0 ± 11.3	100.0 ± 8.34	SFM	100.0 ± 11.9	100.0 ± 10.9
+ $NiCl_2$	133.5 ± 10.9	134.8 ± 17.6	+ 5µg/ml Tf	120.9 ± 10.4	108.3 ± 18.1
+ $SnCl_2$	124.3 ± 10.6	94.53 ± 10.1	+ 84.12µg/ml LA	114.2 ± 12.6	104.5 ± 9.05
+ $CuSO_4$	137.8 ± 15.4	92.81 ± 6.75	+ 1µg/ml PA	107.8 ± 8.46	87.91 ± 11.5
+ MoO_7	133.5 ± 17.8	105.9 ± 4.80	+ 5µg/ml PA	99.39 ± 10.6	90.84 ± 8.86
+ $ZnSO_4$	134.8 ± 13.1	104.3 ± 7.07	+ 10µg/ml PA	77.72 ± 9.97	81.68 ± 6.12
+ All*	122.6 ± 9.84	95.62 ± 6.91	+ 0.01µg/ml HDL	134.6 ± 15.6	63.74 ± 4.39
+ NEAA(1X)	114.6 ± 16.9	118.7 ± 11.5	+ 0.05µg/ml HDL	53.93 ± 9.14	21.97 ± 4.01
+ Na_2SeO_3	144.9 ± 13.7	135.2 ± 8.73	+ 1.0µg/ml HDL	14.45 ± 1.59	4.39 ± 2.19

Results are expressed as the average percentage growth relative to control (SFM*) ± standard deviation (n=8). Acid phosphatase was used as the end point for two separate experiments. The SFM* contained (in addition to McCoys 5a) 10µg/ml insulin, 1.39µg/ml Fe_2SO_4 and 5µg/ml EDTA. Abbreviations: HDL = Bovine derived high density lipoprotein; PA = phosphatidic acid; LPA = lysophosphatidic acid; LA = linoleic acid; All* = all trace elements used by Mendiaz (Appendix J)

Phosphatidic acid had little or no effect on the cells at low concentrations (see above) but became inhibitory at higher concentrations Linoleic acid and transferrin both had little stimulatory effects on the cells The bovine-derived HDL showed conflicting results at the lowest concentration tested (0 01μg/ml) between two experiments At higher concentrations, the HDL became increasingly inhibitory in a dose dependent fashion

The results show that IL-4 had little or no activity while Briclone exhibited variable stimulation between the two assays (maximum of 29 5 to 79 9% stimulation above the control) While Briclone was stimulatory, its entire composition is not defined and its incorporation into the SFM would reduce the defined nature of the medium

Table 3.1.7.4 2 Growth response of NRK cells to IL-4 and Briclone

VARIABLES	ASSAY 1	ASSAY 2
BM	33 00 $\pm$ 4 82	40 20 $\pm$ 5 80
SFM*	100 0 $\pm$ 4 86	100 0 $\pm$ 9 00
+ 0 1% Briclone	113 5 $\pm$ 8 28	122 2 $\pm$ 10 8
+ 0 5% Briclone	129 5 $\pm$ 6 86	144 4 $\pm$ 8 19
+ 1 0% Briclone	124 1 $\pm$ 9 04	172 5 $\pm$ 15 6
+ 5 0% Briclone	119 8 $\pm$ 11 1	179 9 $\pm$ 14 3
+ 0 1U/ml IL-4	99 00 $\pm$ 11 9	82 26 $\pm$ 3 72
+ 0 5U/ml IL-4	102 0 $\pm$ 4 98	109 7 $\pm$ 6 45
+ 1 0U/ml IL-4	107 7 $\pm$ 8 56	116 1 $\pm$ 9 40
+ 5 0U/ml IL-4	94 20 $\pm$ 5 86	114 5 $\pm$ 9 24
+ 10 0U/ml IL-4	75 29 $\pm$ 2 78	106 4 $\pm$ 6 45

Results are expressed as the average percentage growth relative to control (SFM*) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point for two separate experiments The SFM* contained (in addition to McCoys 5a) 10μg/ml insulin, 1 39μg/ml Fe_2SO_4 and 5μg/ml EDTA

The growth factors showed good stimulation (Figure 3 1 7 4 3) ß-FGF alone showed a 2-fold stimulation over the control Although 5ng/ml was best, the stimulation at 1ng/ml was not much less PDGF at 5ng/ml was more stimulatory than ß-FGF, with maximum stimulation of 2 90-fold to 3 47-fold stimulation over the control Although the combination of the two growth factors was not as good as their additive effects, the extent of stimulation was more repeatable between the two assays EDTA was tested again and found to have only a slight positive effect.

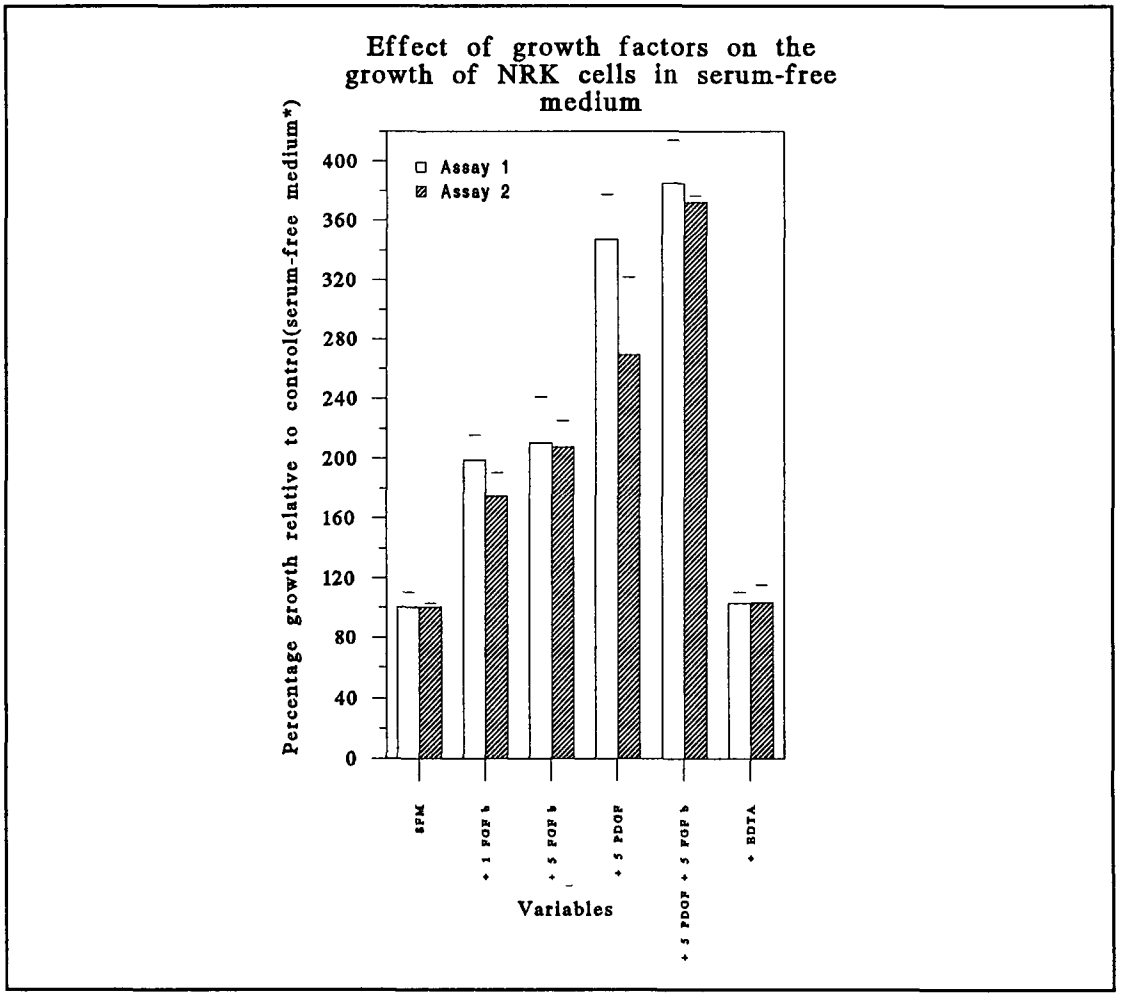


Figure 3.1.7.4.3 shows the growth response of NRK cells to growth factors in serum-free media The results are expressed as the average percentage growth relative to control (serum-free medium*) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 2 separate experiments are shown in Table 3 1 7 4 3 Abbreviations SFM* was 10μg/ml insulin and 1 39μg/ml Fe_2SO_4 in McCoys 5a basal medium

Table 3.1.7.4.3 Effect of growth factors on NRK cells

VARIABLES	ASSAY 1	ASSAY 2
SFM	100 0 ± 10 4	100 0 ± 2 71
+ 1ng/ml ß-FGF	198 8 ± 16 9	174 8 ± 15 9
+ 5ng/ml ß-FGF	210 2 ± 30 9	207 9 ± 17 5
+ 5ng/ml PDGF	346 6 ± 30 4	269 2 ± 52 4
+ 5ng/ml PDGF+ 5ng/ml ß-FGF	384 7 ± 28 9	371 9 ± 4 53
+ EDTA	102 8 ± 7 49	103 3 ± 11 9

When the effects of the trace elements in the absence of Na_2SeO_3 and NEAA were investigated (Table 3 1 7 4 1), there was some differences from the results obtained in the presence of Na_2SeO_3 and NEAA (see Table 3 1 7 2 2) Therefore, from all the experiments carried out, a ratio of the growth achieved in SFM to the growth achieved in basal medium was obtained The ratios for each experiment and the components of each SFM are shown in Table 3 1 7 4 4 By far the best ratio was the combination of 10μg/ml insulin, 1 39μg/ml Fe_2SO_4 and 1μg/ml EDTA

Table 3.1.7.4.4 Comparison of SFM to BM

EXPERIMENT	SFM/BM RATIO	COMPONENTS OF SFM
3 1 6	1 19	Ins/Tf/PGE/HC/T_3
	1 45	Ins/Tf/NEAA/LA/$CaCl_2$/Fe_2SO_4
3 1 7 1	1 096 $\pm$ 0 05	Ins/Fe_2SO_4/EGF/TI/EDTA
3 1 7 2	2 09 $\pm$ 0 17	Ins/Fe_2SO_4/TI/EDTA
3 1 7 3	1 68 $\pm$ 0 39	Ins/Tf/Na_2SeO_3/NEAA/Fe_2SO_4/EDTA
3 1 7 4	2 24 $\pm$ 0 49	Ins/Fe_2SO_4/EDTA

3.1.8 COMPARISON OF SF AND SERUM-SUPPLEMENTED MEDIA

The most stimulatory factors, ß-FGF and PDGF were combined with insulin and Fe_2SO_4 and the growth was compared to the basal medium (McCoys 5a alone) and basal medium supplemented with 1% and 5% DHS In this experiment, duplicate assays were set up in 24-well plates Cell number was used as the end point as determined by haemocytometer counts The results are shown in Table 3 1 8

Table 3.1.8 Comparison of SFM to serum-supplemented medium

VARIABLES	ASSAY 1	ASSAY 2
Basal medium	100 0 ± 20 38	100 0 ± 0 38
Serum-free medium*	1870 6 ± 277 3	2182 3 ± 142 6
Basal medium + 1% DHS	5670 6 ± 26 50	6118 0 ± 283 0
Basal medium + 5% DHS	20612 ± 2096	20000 ± 640 0

Results are expressed as the average percentage growth relative to the basal medium ± standard deviation (n=3)
Abbreviations Serum-free medium* = McCoys 5a supplemented with 10µg/ml insulin, 1 39µg/ml Fe_2SO_4, 1ng/ml 1µg/ml FGF-ß and 5ng/ml PDGF

The results show that the SFM was 20-fold better than the basal medium The basal medium supplemented with 1% DHS was 3-fold as good as the SFM and when the basal medium was supplemented with 5% DHS, it was 10-fold as good as the SFM This SFM was subsequently used to grow NRK cells under serum-free conditions over a 10 passages

3.1.9 SUBCULTURE OF NRK CELLS IN SFM

The true measure of a serum-free medium is the ability to support the growth of cells during sequential passages Such a subculture would show if any residual serum components present in the medium or in the cells were partly responsible for improving the ability of the SFM to support the growth of the cells Two subculture experiments were set up in parallel The variables looked at were McCoys 5a (BM), BM + 10μg/ml insulin + 1 39μg/ml Fe_2SO_4 (=SFM), SFM + 1ng/ml ß-FGF and SFM + 1ng/ml ß-FGF + 5ng/ml PDGF The results are shown in Tables 3 1 9 1 and 3 1 9 2 The subculture procedure is described in Section 2 5 4 Cells were set up at $3x10^4$ cells/well with 2ml of medium per well On subculturing, the action of trypsin was neutralized by trypsin inhibitor After two centrifugation steps, cells were set up in fresh wells

Table 3.1.9.1 Subculture of NRK cells in BM and SFM (Cell Yield $x10^{-4}$/well)

VARIABLES	BM		SFM	
Passage	SUBCULTURE 1	SUBCULTURE 2	SUBCULTURE 1	SUBCULTURE 2
1	0 75 ± 4 51	0 00 ± 0 00	6 12 ± 0 87	4 44 ± 0 26
2	------	------	4 50 ± 3 35	5 92 ± 7 35

Table 3.1.9.2 Subculture of NRK cells in SFM (Cell Yield $x10^{-4}$/well)

VARIABLES	SFM + FGF		SFM + FGF + PDGF	
Passage	SUBCULTURE 1	SUBCULTURE 2	SUBCULTURE 1	SUBCULTURE 2
1	21 33 ± 4 51	17 40 ± 2 12	40 56 ± 1 15	35 31 ± 2 34
2	6 44 ± 0 79	5 81 ± 1 84	2 187 ± 0 09	2 875 ± 0 00
3	9 58 ± 1 58	17 25 ± 0 54	12 87 ± 10 3	32 21 ± 6 24
4	18 56 ± 5 57	19 58 ± 6 26	19 46 ± 2 20	27 06 ± 0 62
5	20 12 ± 4 60	12 46 ± 3 39	35 79 ± 4 66	31 83 ± 1 51
6	22 33 ± 2 88	14 00 ± 1 07	33 03 ± 3 47	25 81 ± 2 56
7	12 75 ± 0 70	15 25 ± 3 70	5 06 ± 1 67	14 43 ± 1 50
8	57 18 ± 8 66	30 94 ± 3 97	50 72 ± 8 62	55 22 ± 3 84
9	19 37 ± 1 56	14 21 ± 1 63	59 62 ± 9 01	29 27 ± 8 30
10	18 91 ± 1 12	------	69 50 ± 9 89	------
11	20 62 ± 0 53	------	46 50 ± 5 63	------

Results in both tables are expressed Cell yield per well ($x10^{-4}$) ± standard deviation (n=3)

The results show that the SFM when supplemented with FGF or FGF and PDGF could support the growth of NRK cells in SFM, although the growth was low at times The basal medium alone could not support growth In the SFM (McCoys 5a + 10μg/ml insulin + 1 39μg/ml Fe_2SO_4), growth was low and only continued through two passages, becoming increasingly variable in its ability to support growth For the more complex SFM (incorporating ß-FGF or ß-FGF and PDGF) growth continued until the end of the experiment After inoculation at passage 10, assay 2 became contaminated In general growth was better in subculture 1 than subculture 2

In the second passage, the growth was very low This may have been due to the cells properly adapting to serum-free conditions Up until passage 7, cells were fed every 4 days with fresh medium When this was then stopped a big increase of nearly 2-fold relative to earlier passages was seen in the SFM containing FGF and PDGF Although an increase was seen in passage 7 for the SFM containing ß-FGF, it was not maintained through to further passages, while continued high growth activity in the SFM with ß-FGF and PDGF was seen This may indicate the presence of some autocrine activity for the NRK cells It was interesting that this increased activity only occurred with the combination of FGF and PDGF In addition, at passages 9 - 11, it was observed that the medium containing FGF and PDGF had much fewer cells floating in suspension than in the SFM with FGF only

In conclusion for this section, we set out to grow NRK cells in a SFM described in the literature (Rizzino, 1984) for the NRK subclone 49F When the NRK cells would not grow in this SFM, it was decided to develop a new SFM Thus two media have been developed to grow NRK cells in low serum and serum-free medium In low serum, 1% DHS supplemented with 0 5mg/ml BSA, 5μg/ml insulin, 5μg/ml transferrin and 1ng/ml EGF, was more stimulatory than 5% FCS In serum-free medium, McCoys 5a supplemented with 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 1ng/ml ß-FGF and 5ng/ml PDGF, was capable of supporting the growth of NRK cells at low initial cell densities through 10 passages However, the extent of growth was much lower than that obtained in the serum-supplemented medium (10-fold less as seen in section 3 1 8)

3.2 DEVELOPMENT OF SERUM-FREE ASSAY SYSTEMS

Two new cell lines were introduced into the investigations, Madin-Darby Canine Kidney (MDCK) and Chinese Hamster Ovary (CHOK1) cells For both cell lines, serum-free media have been reported It was hoped to use these established serum-free systems to investigate the possibility of replacing animal-derived proteins with either recombinant proteins or with non-ammal-derived compounds For MDCK, a SFM was developed by Taub *et al* in 1979 The most important components in the medium were found to be transferrin and prostaglandin This therefore appeared to be a suitable system to study the replacement of transferrin However, before this could be done it was necessary to ensure that a suitable assay system was used which could detect the effects of varying transferrin or its concentrations in serum-free medium All subsequent investigations with MDCK refer to the SFM described by Taub in 1979

For CHOK1 cells several serum-free media were reported (Mendiaz *et al* , 1986, Hamilton and Ham, 1977 and Darfler, 1990) Some investigations found that insulin-independent mutants developed in serum-free medium spontaneously even though insulin was incorporated into the SFM (Mendiaz *et al* , 1986) Transferrin was found not to be necessary but was still incorporated into the SFM since it had some stimulatory effect at low concentrations of Fe_2SO_4 If the insulin and transferrin could either be removed or replaced by recombinant products or non animal-derived compounds, the SFM would be devoid of any animal-derived component It was necessary first to get the cells to grow under serum-free conditions, before discovering whether any of these systems were suitable for further investigations

3.2.1 MDCK CELLS IN SFM

The serum-free medium initially described by Taub *et al* in 1979 uses ATCC as the basal medium (ATCC = 1 1 vol/vol DMEM Ham's F12) The medium was buffered with 15mM HEPES and 11mg/ml $NaHCO_3$ The additional factors were 10nM Na_2SeO_3, 5μg/ml insulin, 5μg/ml transferrin, 25ng/ml prostaglandin E_1, 5pM tri-iodothyromne and 50nM Hydrocortisone This SFM had been used already in this laboratory by Edel Murphy (1986) She found that while the SFM promoted growth in open systems, problems existed when attempts were made to subculture the cells in tissue culture flasks

3.2.1.1 Growth response of MDCK cells in SFM

A growth curve experiment was set up to find out how effectively the serum-free medium worked Three parameters were investigated over a period of 8 days basal medium, SFM and serum-supplemented medium The basal medium consisted of ATCC supplemented with HEPES, $NaHCO_2$ and Na_2SeO_3 as above and l-glutamine The serum-supplemented medium consisted of ATCC, 7 5% $NaHCO_2$, 10mM HEPES, 5% FCS (#109) and l-glutamine Cells were set up at $5x10^3$ cells/well in 24-well plates Triplicate assays of SFM and serum-supplemented medium (SSM) were carried out, with only duplicates of the basal medium After several intervals of time the plates were taken down and the viable cell number per well was determined using a haemocytometer Cell counts were chosen as no comparison of image analysis or acid phosphatase had been carried out The results are shown in Figure 3 2 1 1

Over a period of 8 days the basal medium could not support cell growth and division It did however, support cell viability After a lag phase of 4 to 5 days in the SFM, the cell number began to increase and on day 8 showed a slightly higher cell number than that obtained in the SSM on day 8 The SSM also showed a lag phase of about 2 days, after which exponential growth appeared until day 5 Between day 5 and 8, the rate of growth appeared to have decreased

These results show that the SFM described by Taub supported the growth of MDCK cells The growth was initially slower than in SSM but by day 8, good growth was obtained The observation that growth in SFM was better than in SSM by day 8 may have been due to the possibility of the cells in SSM being in a decline phase Peak growth in SSM probably occurred between days 5 and 8

Table 3 2.1.1 Growth curve of different media (Cells per well $x10^{-4}$)

SSM	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	1 900 $\pm$ 0 44	1 760 $\pm$ 0 451	1 933 $\pm$ 1 069
Day 3	5 952 $\pm$ 0 88	5 967 $\pm$ 1 136	5 560 $\pm$ 0 950
Day 4	15 60 $\pm$ 0 14	19 56 $\pm$ 2 800	20 28 $\pm$ 2 940
Day 5	30 97 $\pm$ 4 76	33 80 $\pm$ 6 210	33 83 $\pm$ 4 040
Day 8	43 45 $\pm$ 1 91	43 70 $\pm$ 7 562	38 30 $\pm$ 0 608

Results are expressed as cell number ($x10^{-4}$) $\pm$ standard deviation (n=3) Abbreviations SSM = Serum-supplemented medium

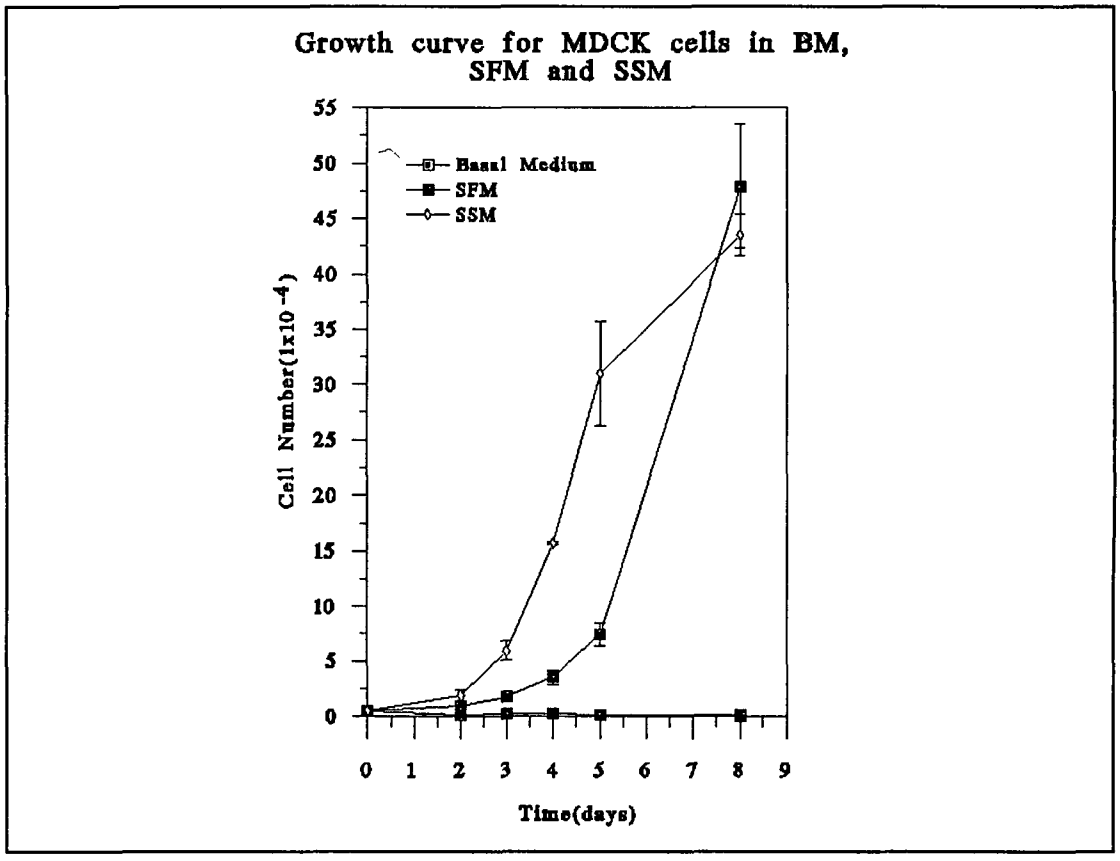


Figure 3.2.1.1 shows the growth curve for MDCK cells in basal medium, serum-free and serum-supplemented media Results are expressed as cell number ($x10^{-4}$) ± standard deviation (n=3) All assays were set up with an initial cell concentration of 0 $5x10^4$ per well Cell number was determined by haemocytometer counts Abbreviations BM = basal medium, SFM = serum-free medium, SSM = serum-supplemented medium Results are shown in 3 2 1 1

Table 3.2.1.1continued Growth curve of different media (Cells per well $x10^{-4}$)

BM	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	0 147 ± 0 002	0 173 ± 0 016	----------
Day 3	0 280 ± 0 058	0 200 ± 0 086	----------
Day 4	0 283 ± 0 150	0 160 ± 0 290	----------
Day 5	0 117 ± 0 104	0 133 ± 0 029	----------
Day 8	0 066 ± 0 028	----------	----------
SFM	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	1 100 ± 0 12	0 850 ± 0 071	0 930 ± 0 416
Day 3	1 460 ± 0 37	0 383 ± 0 100	1 800 ± 1 000
Day 4	3 450 ± 0 26	1 050 ± 0 070	3 525 ± 0 671
Day 5	6 300 ± 2 20	2 100 ± 0 141	7 420 ± 1 068
Day 8	45 60 ± 4 31	10 36 ± 1 930	47 86 ± 5 580

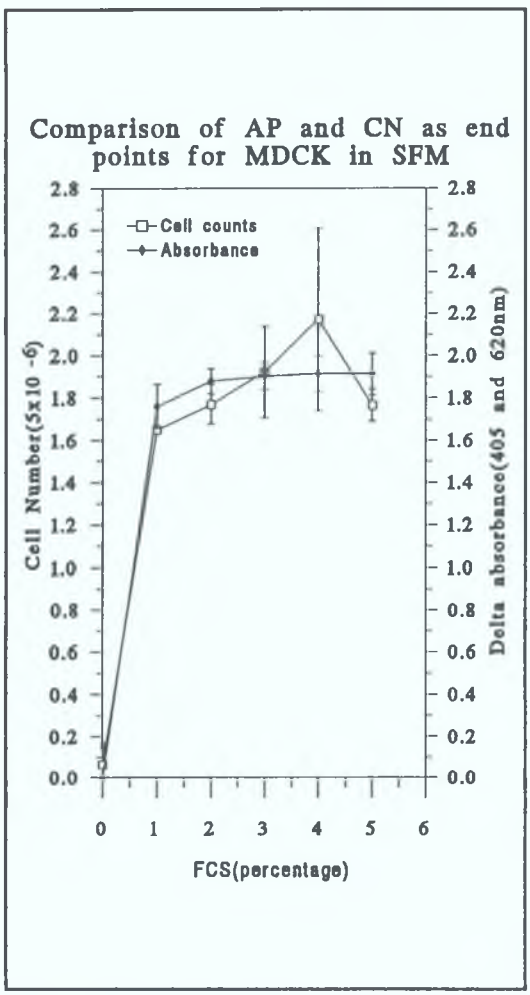
3.2.1.2 Acid Phosphatase as an end point for MDCK assays

To use acid phosphatase as an end point for studying the growth response of MDCK cells in serum-free medium, an experiment was carried out to compare growth in a serum dilution curve with acid phosphatase production (Figure 3 2 1 2 1) It was hoped that if MDCK cells showed a similar response to serum dilutions and acid phosphatase, the whole assaying system could be miniaturized This would mean that a lot more variables could be screened rapidly 24- and 96-well plates were set up with serum dilutions The 24-well plates were trypsimzed and cell counts were made using a haemocytometer, while acid phosphatase was used as the end point for the 96-well plates

Microscopic observations before taking down assays revealed little difference in the extent of growth for the higher serum concentrations (2 - 5%FCS) When cell number and acid phosphatase were used as end points, a non linear response in relation to serum concentration was obtained After 1% FCS, neither end point showed increased growth with increased serum concentration The responses of both end points were similar, indicating (indirectly) the suitability of acid phosphatase (AP) to reflect changes in cell numbers

To confirm the ability of AP to reflect changes in cell number directly, a cell dilution curve was made up in 5% FCS 8 x 100μl of each cell suspension was aliquoted and allowed to attach to a 96-well plate (approximately 6 hours) Microscopic observations showed that the cells had attached and had not started to divide The assay was then taken down with acid phosphatase (AP) as the end point The results are illustrated in Figure 3 2 1 2 2 and show a linear response between AP production and cell number

Both methods showed that acid phosphatase exhibited a linear relationship to cell number in the range tested This meant that subsequent experiments could be miniaturized to 96-well plates This would allow a greater range of variables to be tested with a smaller number of cells, a smaller amount of reagents and less time



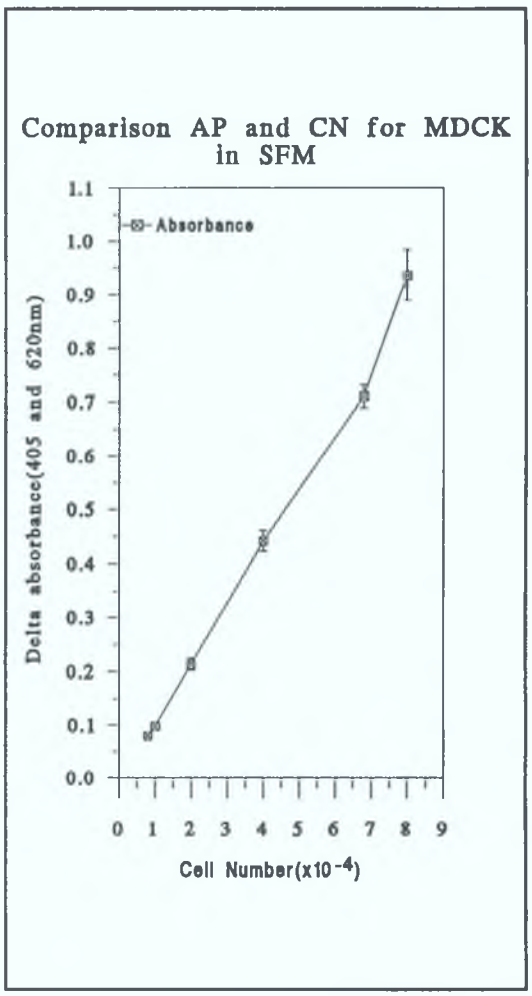


Figure 3.2.1.2.1 and 2 show the comparison of acid phosphatase (AP) and cell number (CN) for MDCK cells. The results are expressed as the average (absorbance or cell number) ± standard deviation (n=3 for cell number in Figure 3.2.1.2.1 and n=8 for absorbance values). Only one experiment was carried out using increasing serum concentrations (indirect comparison). Three separate assays were carried out for the direct comparison of AP production versus cell number. The results are shown in Table 3.2.1.2.1.

Table 3.2.1.2.1 Comparison of AP and CN as end points for MDCK in SFM

INDIRECT COMPARISON OF CN AND AP (VIA SERUM CONCENTRATION)			DIRECT COMPARISON OF CN AND AP			
FCS	AP	CN ($5x10^{-6}$)	CN (10^{-5})	ASSAY 1	ASSAY 1	ASSAY 2
0% FCS	0.000 ± 0.000	0.065 ± 0.01	0.08	0.084 ± 0.003	0.080 ± 0.003	0.074 ± 0.003
1% FCS	1.760 ± 0.102	1.652 ± 0.05	0.10	0.111 ± 0.007	0.098 ± 0.007	0.096 ± 0.007
2% FCS	1.879 ± 0.059	1.770 ± 0.09	0.20	0.219 ± 0.016	0.213 ± 0.011	0.198 ± 0.012
3% FCS	1.902 ± 0.064	1.923 ± 0.21	0.40	0.516 ± 0.021	0.442 ± 0.019	0.412 ± 0.045
4% FCS	1.914 ± 0.084	2.173 ± 0.43	0.68	0.718 ± 0.060	0.711 ± 0.021	0.676 ± 0.015
5% FCS	1.914 ± 0.100	1.760 ± 0.07	0.80	1.110 ± 0.045	0.936 ± 0.047	0.904 ± 0.067

3.2.1.3 Effect of insulin on MDCK cells in SFM

Initial studies were carried out to investigate the response of MDCK cells to varied concentrations of bovine insulin The results are shown in Figure 3 2 1 3 Assays were set up so that the only variable component of the SFM was insulin

The results show that increasing concentrations of insulin resulted in increased growth up to approximately 5 to 10μg/ml where maximum activity was seen The extent of stimulation varied between 1 9-fold and 2 5-fold over the control (no insulin) In all (discounting standard deviations) assays, the extent of stimulation decreased between 10 to 20μg/ml

3.1.2.4 Effect of transferrin on MDCK cells in SFM

Initial studies were carried out to see how responsive MDCK cells were to bovine transferrin Taub (1979) reported that transferrin was the second most important component in the serum-free medium after prostaglandin The results are shown in Figure 3 2 1 4 Assays were set up so that transferrin was the only variable component in the SFM

The results showed that increasing concentrations of transferrin resulted in increased growth Maximum stimulation occurred at 5 - 20μg/ml The extent of maximum stimulation varied between assays, 1 37-fold to 4-fold stimulation over the control (no transferrin) In contrast with the results obtained for insulin, no loss of stimulation was seen at the highest concentration tested (20μg/ml)

3.2.1.5 Effect of bovine serum albumin on MDCK cells in SFM

The effects of BSA fraction V and fatty acid free on the growth of MDCK cells in SFM were investigated (Figure 3 2 1 5) In three of four assays, BSA fatty acid free was marginally more stimulatory than BSA fraction V Both showed stimulation at the lower concentrations (0 1 and 0 5mg/ml) but the extent of stimulation was low, only reaching a maximum of 24 2 to 52 6% for fraction V and 33 2 - 63 9% for fatty acid free growth above the control (SFM, no added BSA) At higher concentrations both became increasingly inhibitory BSA fraction V was more inhibitory than BSA fatty acid free at these concentrations and marginally less stimulatory at the lower concentrations

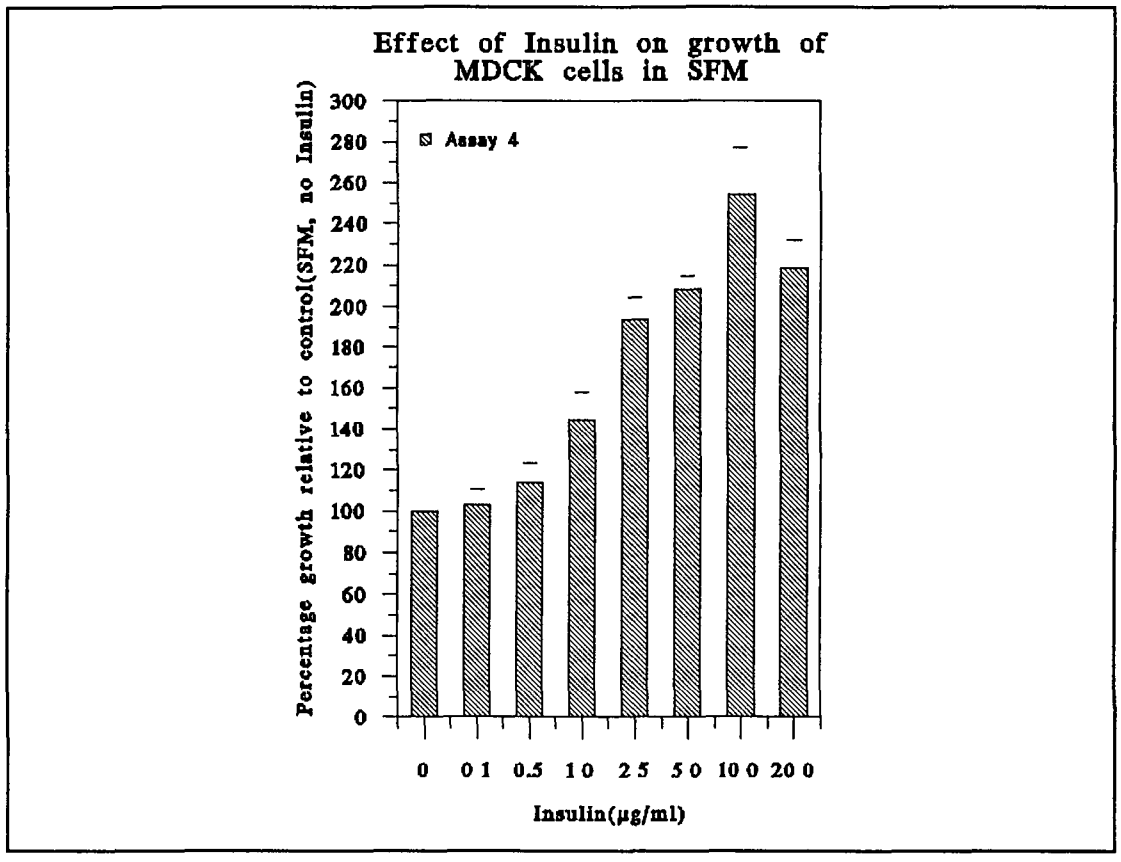


Figure 3.2.1.3 shows the effect of insulin on MDCK cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments Results for 4 separate experiments are shown in Table 3 2 1 3

Table 3.2.1.3 Effect of insulin (µg/ml) on MDCK cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 7 96	100 0 ± 6 33	100 0 ± 7 28	100 0 ± 0 01
0 1µg/ml	117 0 ± 11 3	104 4 ± 8 46	103 3 ± 12 2	103 0 ± 7 65
0 5µg/ml	162 4 ± 15 6	117 7 ± 10 3	126 8 ± 9 62	113 6 ± 10 0
1 0µg/ml	199 6 ± 20 9	132 7 ± 7 02	185 7 ± 19 3	144 6 ± 13 4
2 5µg/ml	228 2 ± 14 7	150 5 ± 10 7	208 7 ± 16 4	193 5 ± 10 8
5 0µg/ml	252 3 ± 20 7	179 2 ± 13 0	217 3 ± 17 4	208 4 ± 6 10
10 0µg/ml	233 7 ± 27 1	190 7 ± 18 8	215 7 ± 16 9	254 3 ± 23 0
20 0µg/ml	206 9 ± 17 9	141 8 ± 10 7	150 2 ± 12 7	218 5 ± 13 6

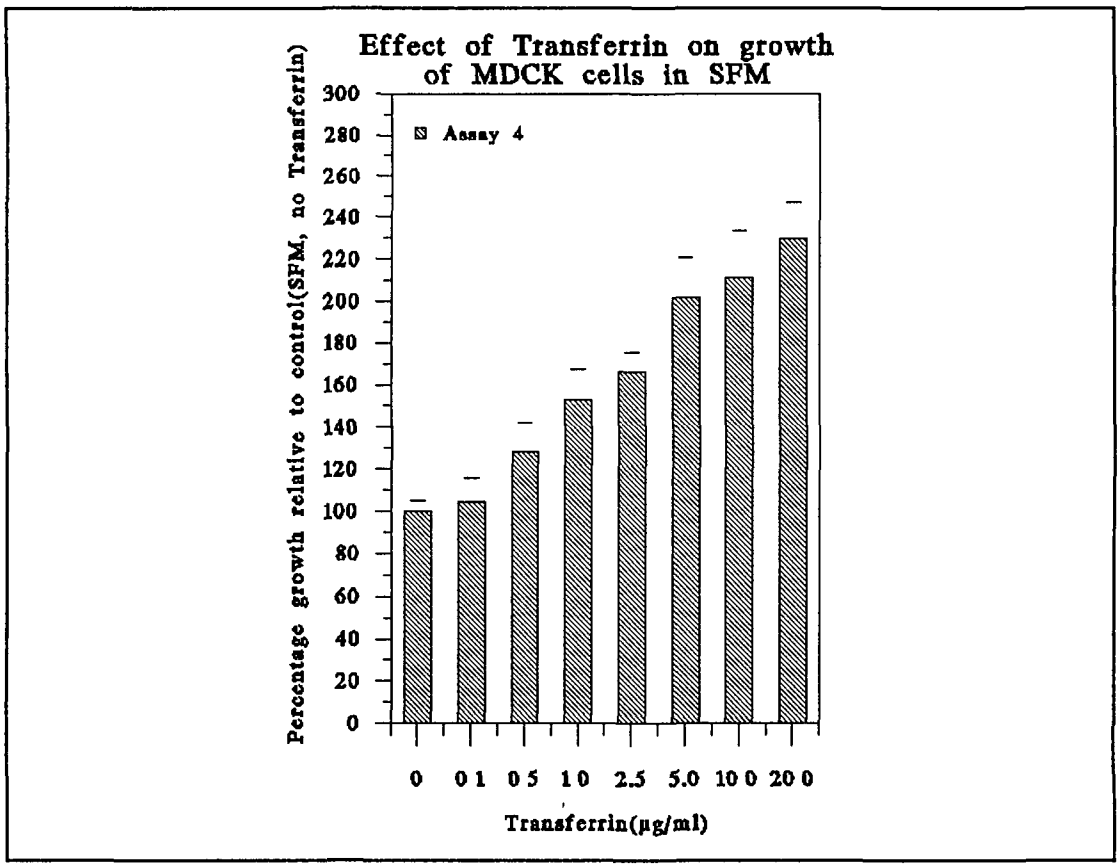


Figure 3.2.1.4 shows the effect of transferrin on MDCK cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments Results for 4 separate experiments are shown in Table 3 2 1 4

Table 3.2.1.4 Effect of transferrin (µg/ml) on MDCK cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 11 8	100 0 ± 3 95	100 0 ± 9 57	100 0 ± 5 08
0 1µg/ml	130 5 ± 10 7	100 8 ± 7 39	110 0 ± 9 22	104 3 ± 11 4
0 5µg/ml	238 6 ± 20 2	125 2 ± 9 22	122 8 ± 6 69	128 2 ± 13 8
1 0µg/ml	273 1 ± 16 6	127 8 ± 8 70	168 9 ± 14 2	152 8 ± 14 6
2 5µg/ml	310 8 ± 14 3	135 9 ± 7 79	175 9 ± 15 2	165 7 ± 9 80
5 0µg/ml	317 5 ± 22 9	137 9 ± 9 87	214 2 ± 20 1	202 1 ± 18 9
10 0µg/ml	408 1 ± 14 6	136 9 ± 9 48	230 9 ± 17 3	211 4 ± 21 5
20 0µg/ml	405 0 ± 19 3	130 3 ± 10 1	232 8 ± 18 3	229 1 ± 17 9

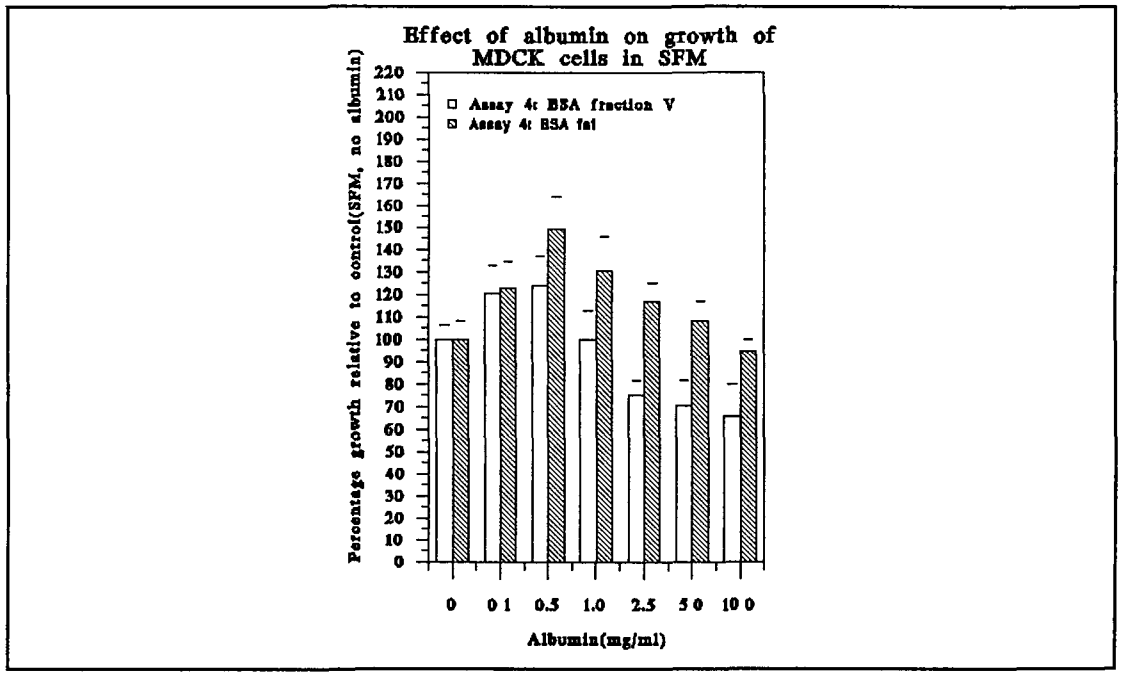


Figure 3.2.1.5 shows the effect of BSA fraction V and fatty acid free on MDCK cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no BSA) ± standard deviation (n=8) Abbreviations Con = Control (no BSA) Acid phosphatase was used as the end point for experiments Results for 4 separate experiments are shown in Tables 3 2 1 5a and b

Table 3.2 1.5a Effect of BSA fraction V (mg/ml) on MDCK cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 3 30	100 0 ± 7 80	100 0 ± 6 21	100 0 ± 7 66
0 1mg/ml	152 6 ± 9 24	116 8 ± 9 87	120 5 ± 12 5	129 2 ± 7 36
0 5mg/ml	138 9 ± 10 3	125 4 ± 16 6	124 2 ± 12 9	105 1 ± 9 44
1 0mg/ml	111 7 ± 9 88	107 0 ± 11 6	100 0 ± 13 0	84 46 ± 7 49
2 5mg/ml	84 59 ± 6 16	79 00 ± 34 4	75 18 ± 6 81	34 05 ± 6 09
5 0mg/ml	58 70 ± 4 17	100 0 ± 5 60	70 54 ± 11 1	9 620 ± 0 48
10 0mg/ml	30 65 ± 3 94	85 83 ± 5 60	65 89 ± 13 9	0 010 ± 0 24

Table 3.2.1.5b Effect of BSA fatty acid free (mg/ml) on MDCK cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 17 5	100 0 ± 6 69	100 0 ± 8 40	100 0 ± 5 56
0 1mg/ml	163 9 ± 18 2	89 46 ± 5 77	122 8 ± 11 5	133 2 ± 5 46
0 5mg/ml	145 2 ± 9 04	136 4 ± 10 4	149 1 ± 14 9	113 9 ± 8 66
1 0mg/ml	116 8 ± 7 84	156 5 ± 7 90	130 7 ± 14 8	93 52 ± 7 51
2 5mg/ml	79 47 ± 6 88	159 9 ± 7 15	117 2 ± 7 95	52 20 ± 3 38
5 0mg/ml	58 38 ± 4 27	137 3 ± 10 1	108 2 ± 8 96	37 31 ± 1 73
10 0mg/ml	42 01 ± 3 10	110 6 ± 9 80	94 60 ± 5 18	21 98 ± 3 23

3.2.1.6 Subculture of MDCK cells in SFM

The results described above confirm that MDCK cells grow well in SFM However, the real test of a SFM is the ability of the medium to support growth and proliferation over a period of time Figure 3 2 1 6 shows the result of triplicate experiments in which three flasks per assay were subcultured 9 times After the first 4 days in serum-free medium, cells appeared 90% confluent, so subsequent subcultures were carried out every 4 days For each experiment, 400ml of SFM was made up These were aliquoted out for each subculture and frozen at -20°C Sufficient media was then thawed down slowly at 4°C before use Cell counts were determined using a haemocytometer Fresh flasks were inoculated with $2x10^5$ cells per flask at each subculture

In an initial experiment, MDCK cells were set up in closed flasks The medium turned basic and the cells would not grow (previously reported by Edel Murphy, 1986) However, in an open system as seen with petri dishes, 24-well and 96-well plates, good growth occurred There was obviously something causing the pH to rise It may be that either HEPES or sodium bicarbonate is inhibitory to MDCK cells under closed serum-free conditions (as in a closed buffered system) Indeed HEPES has been found to be inhibitory to some cells under serum-free conditions (Bowman *et al* , 1985, Bell and Quinton, 1991) It may also be that, under closed serum-free conditions, either of or both of the buffers could react adversely with some other component of the serum-free medium As there were many possible reasons for the change in pH, it was decided to try vented flasks The cells grew well, so it was decided to use vented flasks in the subculture experiment

Microscopic observations showed no significant difference between cells in serum-free medium and serum-supplemented medium In addition, growth was almost as good as that seen with 5% FCS This was observed in the first three subcultures as flasks with serum containing media required trypsinizing every three to four days However, no cell count was made

The results show that the cells did not appear to need a period of adaption to grow in SFM The cells were passaged 9 times over a period of 40 days (subcultured at $2x10^5$ cells per $25cm^2$ flask every four days) This meant that the cells could be grown in SFM for several passages before being used in an assay which in turn meant that no carry over from the serum could affect the experiment

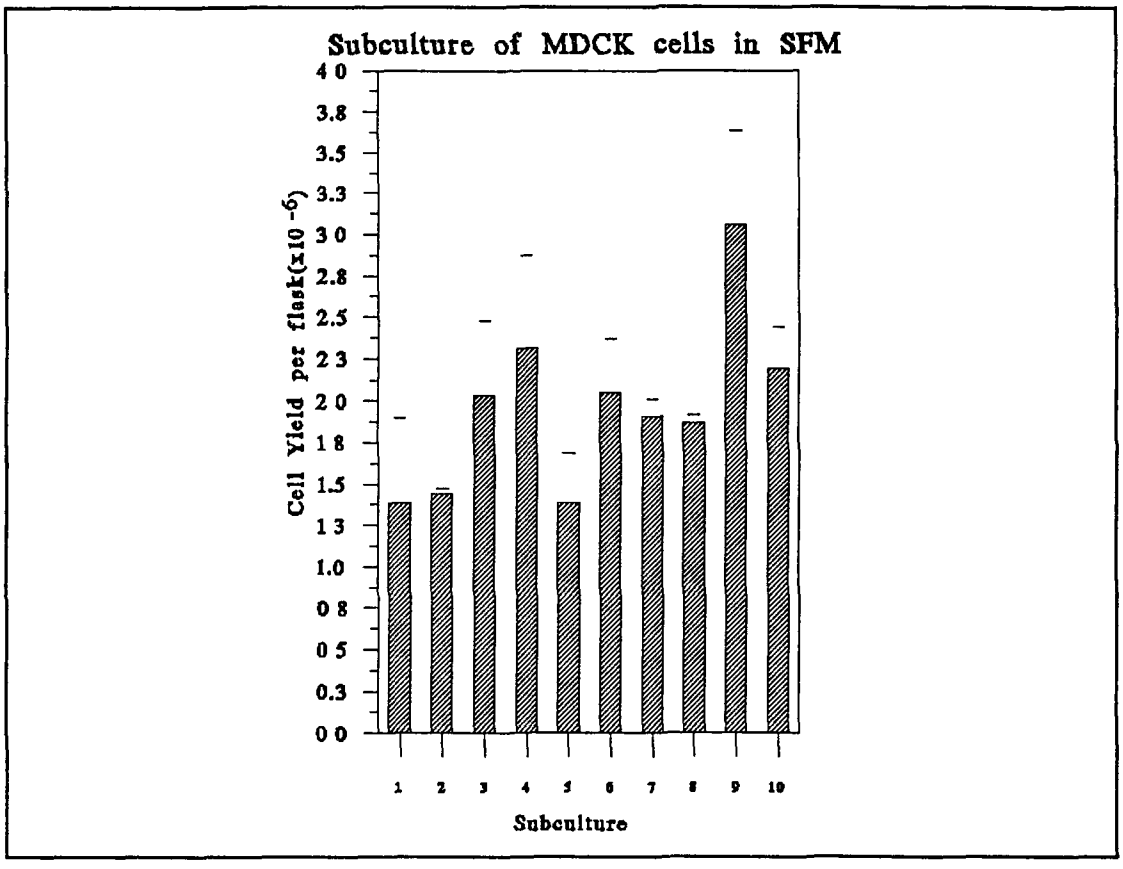


Figure 3.2.1.6 shows the subculture of MDCK cells in serum-free medium Results are expressed average cell yield per flask ± standard deviation (n=3) Results for 3 separate subcultures are shown in Table 3 2 1 6

Table 3.2.1.6 Subculture of MDCK cells in SFM (Cell Yield x10 ^{6}/flask)

SUBCULTURE	ASSAY 1	ASSAY 2	ASSAY 3
1	1 115 ± 0 247	1 940 ± 0 056	5 110 ± 0 010*
2	1 450 ± 0 056	1 430 ± 0 020	2 730 ± 0 389*
3	1 790 ± 0 205	0 516 ± 0 315	2 280 ± 0 523
4	1 800 ± 1 098	2 770 ± 0 140	0 980 ± 0 099
5	1 597 ± 0 287	1 535 ± 0 268	1 260 ± 0 115
6	2 265 ± 0 418	2 130 ± 0 061	1 747 ± 0 150
7	1 860 ± 0 085	2 060 ± 0 440	2 256 ± 0 006
8	1 855 ± 0 061	1 877 ± 0 046	———
9	3 336 ± 0 429*	2 435 ± 0 346	———
10	1 970 ± 0 137	2 266 ± 0 380	———

Results are shown as the Cell yieldx10^{6} per flask for three separate * indicates where the subculture was left for 5 days before passaging In assay 3, passage 8 became contaminated

In summary, the results show that MDCK cells could be grown in the SFM described by Taub (1979) The assay procedure was miniaturized with acid phosphatase as the end point Varying both insulin and transferrin showed growth response and indicated that both could be used as a basis for studying the removal of animal-derived products from SFM Albumin, either BSA fraction V or fatty acid free, had very little stimulatory effect at low concentrations and became increasingly inhibitory at higher concentrations

The MDCK cells were subcultured in SFM for 9 passages and showed no significant adaption period The ability of the cells to grow for many passages in serum-free medium meant that the cells could be grown for one passage in SFM before assaying for insulin or transferrin replacements This would ensure the absence of residual serum contaminants (present inside the cells) which could otherwise interfere with results

3.2.2 CHOK1 CELLS IN SFM

Many SFM have been cited to support the growth of CHOK1 cells Ham's F12 was originally designed to support the clonal growth of such cells in defined medium (Ham, 1965) Later, however, this basal medium on its own was found not to be sufficient to support growth (Hamilton and Ham, 1977) For the present studies, a modified Ham's F12 developed by Mendiaz *et al* (1986) was assessed Ham's F12 was buffered by 1 176 g/l $NaHCO_3$ and 7 55 mM HEPES The medium was further supplemented with $3x10^{-2}$ μM Na_2SeO_3, 5μM $FeSO_4$, 5μg/ml transferrin, 10μg/ml insulin, 0 3μM linoleic acid, $1x10^{-2}\mu$M NEAA and $6x10^{-2}\mu$M $CaCl_2$ The trace elements quoted by Mendiaz (Appendix J) were omitted initially as these were added only to ensure reproducibility between different batches of Ham's F12

3.2.2.1 Growth response of CHOK1 cells in SFM

Several attempts were made to get CHOK1 cells to grow in the SFM described The cells did not attach and spread Two attachment factors, fibronectm and lamimn, were used to try and increase attachment, but no improvement was seen This suggested that after trypsinizmg and the initial centrifugation, some residual trypsin may have remained in the medium preventing proper attachment Two centrifugation steps were introduced In the first step, trypsin inhibitor was added to the trypsinized cells and centrifuged at 1000rpm for 5 minutes The supernatant was removed, the pellet was resuspended in basal medium only and spun as before This time the cells attached and grew in the SFM both with and without fibronectin as an attachment factor Consequently, two centrifugation steps became part of the standard procedure for setting up CHOK1 cells in SF experiments

A growth curve was set up to compare growth m basal medium, serum-free and serum-supplemented medium Fibronectin was used as the attachment factor, with several plates as controls without fibronectin The experiment was carried out in 24-well plates with three wells per variable in three separate assays The plates were set up with $5x10^3$ cells per well The cells were trypsinized in 100μl TV until detached and 100μl of 5% FCS in basal medium was added to stop the action of trypsin Cell counts were determined using a haemocytometer The results are shown in Figure 3 2 2 1

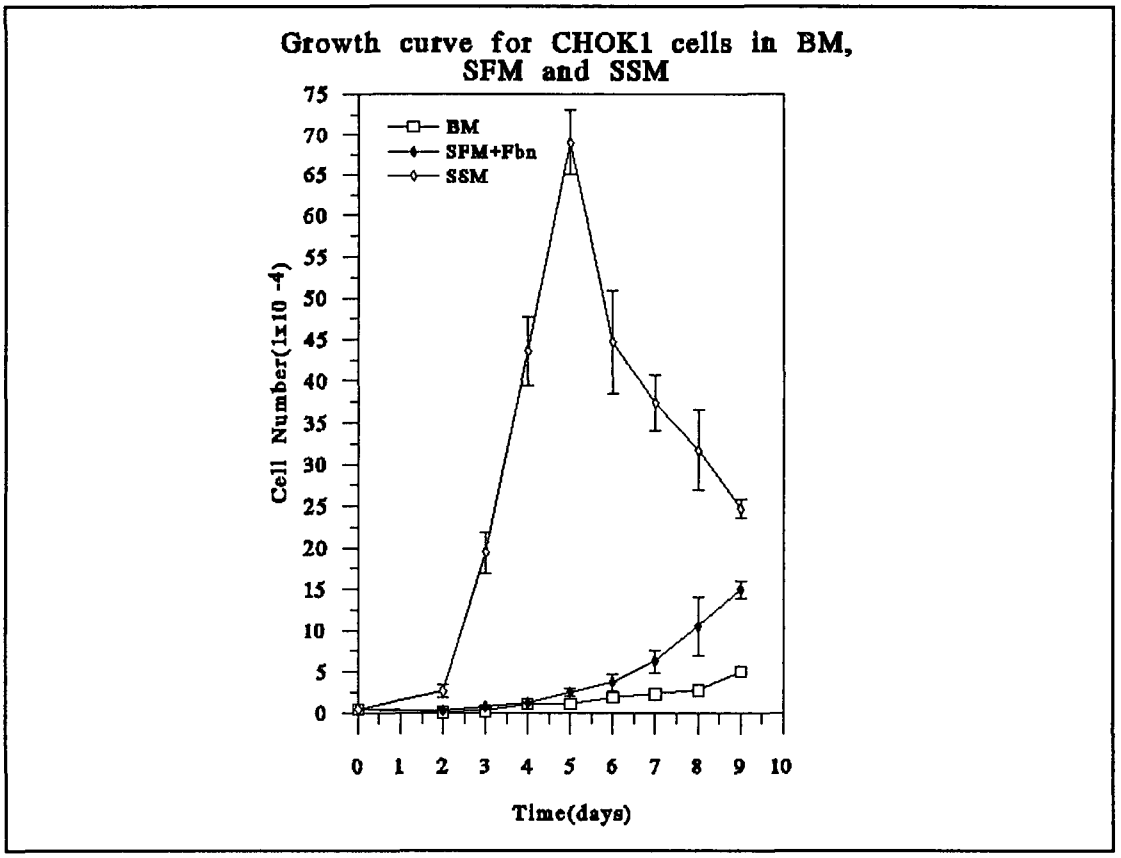


Figure 3.2.2.1 shows the growth curve for CHOK1 cells in basal medium, serum-free and serum-supplemented media Results are expressed as the average cell number ($x10^{-4}$) $\pm$ standard deviation (n=3) All assays were set up with an initial cell concentration of 0 $5x10^4$ per well Cell number was determined by haemocytometer counts Results for two to three separate experiments are shown in Tables 3 2 2 1a to d Abbreviations BM = basal medium, SFM + Fbn = serum-free medium + fibronectin, SSM = serum-supplemented medium, SFM = SFM without attachment factors, Fbn = fibronectin

Table 3.2.2.1a Growth curve of different media (Cells per well $x10^{-4}$)

BM	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	0 06 $\pm$ 0 058	0 083 $\pm$ 0 029	----------
Day 3	0 33 $\pm$ 0 189	0 220 $\pm$ 0 161	----------
Day 4	1 47 $\pm$ 0 035	1 066 $\pm$ 0 115	----------
Day 5	1 85 $\pm$ 0 212	1 130 $\pm$ 0 153	----------
Day 6	3 86 $\pm$ 0 534	1 924 $\pm$ 0 460	----------
Day 7	4 06 $\pm$ 1 030	2 300 $\pm$ 0 150	----------
Day 8	6 90 $\pm$ 0 710	2 750 $\pm$ 0 210	----------
Day 9	10 0 $\pm$ 0 560	5 100 $\pm$ 0 452	----------

Table 3.2.2.1b Growth curve of different media (Cells per well $x10^{-4}$)

SSM	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	2 450 ± 0 53	2 65 ± 0 74	2 400 ± 0 95
Day 3	27 20 ± 2 29	19 40 ± 2 42	20 80 ± 3 15
Day 4	53 40 ± 3 75	43 60 ± 4 18	53 40 ± 5 55
Day 5	65 55 ± 2 04	69 00 ± 4 01	70 28 ± 20 5
Day 6	43 08 ± 1 23	44 67 ± 6 20	46 75 ± 2 88
Day 7	38 08 ± 7 75	37 42 ± 3 30	35 61 ± 16 8
Day 8	31 00 ± 3 12	31 75 ± 4 92	31 50 ± 6 38
Day 9	22 58 ± 2 67	24 75 ± 1 09	26 75 ± 5 02

Table 3.2.2.1c Growth curve of different media (Cells per well $x10^{-4}$)

SFM + Fbn	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	0 266 ± 0 11	0 366 ± 0 03	0 383 ± 0 16
Day 3	0 716 ± 0 21	0 730 ± 0 22	0 800 ± 0 09
Day 4	2 900 ± 0 98	1 233 ± 0 06	2 060 ± 1 08
Day 5	3 329 ± 1 99	2 530 ± 0 51	3 800 ± 1 21
Day 6	6 400 ± 0 66	3 700 ± 1 08	3 400 ± 0 28
Day 7	11 45 ± 0 71	6 250 ± 1 34	6 060 ± 1 49
Day 8	16 80 ± 0 42	10 50 ± 3 61	10 67 ± 1 06
Day 9	19 02 ± 1 80	14 97 ± 1 08	13 70 ± 0 71

Table 3.2 2 1d Growth curve of different media (Cells per well $x10^{-4}$)

SFM w/o Fbn	ASSAY 1	ASSAY 2	ASSAY 3
Day 2	0 475 ± 0 18	1 810 ± 0 68	1 700 ± 0 26
Day 3	0 266 ± 0 19	3 775 ± 0 11	3 800 ± 0 56
Day 7	----------	42 00 ± 2 23	38 86 ± 1 09

The basal medium supported cell viability Proliferation only occurred after prolonged incubation (days 7 to 9) In 5% FCS-supplemented medium, cell growth increased to a maximum of $6x10^5$ on day 5 and decreased to $4X10^5$ cells/well on day 6

In the SFM with fibronectin, the growth did not appear to be much better than in the basal medium The cell number increased on days 7 to 9 to approximately double that in the basal medium

In a control plate without fibronectin, the growth was surprisingly better Microscopically, in wells without an attachment factor, some of the cells appeared more rounded up (as was seen in the serum-supplemented medium) As the growth without fibronectin appeared to be so good, it was decided to omit an attachment factor in future experiments The control without fibronectin was only carried out on 3 days The results for the SFM without fibronectin are shown in Table 3 2 2 1d

3.2.2.2 Acid Phosphatase as an end point for CHOK1 cells in SFM

To use acid phosphatase (AP) as an end point for experiments using CHOK1 cells in SFM, it was necessary to establish a linear relationship between cell number and AP production The assay was set up as in section 3 2 1 2 2 A linear response was obtained (Figure 3 2 2 2), which meant that AP could be used as an end point for subsequent experiments

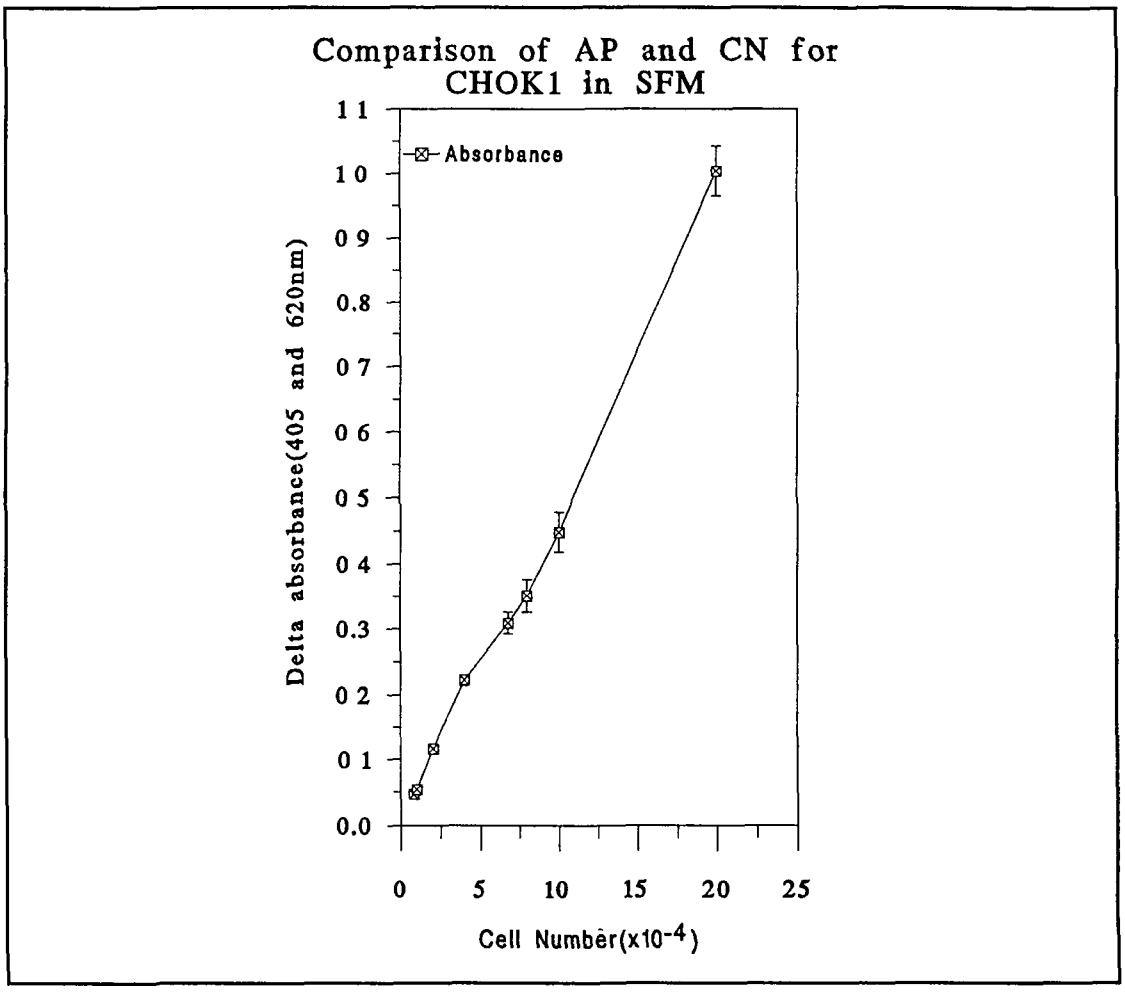


Figure 3.2.2.2 shows the comparison of acid phosphatase (AP) and cell number (CN) for CHOK1 cells in serum-free medium (SFM) The results are expressed as the average (absorbance) ± standard deviation (n=8) Three separate assays were carried out for the direct comparison of AP production versus cell number The results are shown in Table 3 2 2 2

Table 3.2.2.2 Comparison of AP and CN as end points for CHOK1 in SFM

CELL NUMBER x10^{-4}	ASSAY 1	ASSAY 2	ASSAY 3
0 08	0 047 ± 0 004	0 050 ± 0 004	0 047 ± 0 003
0 10	0 055 ± 0 004	0 065 ± 0 005	0 058 ± 0 007
0 20	0 117 ± 0 007	0 129 ± 0 013	0 108 ± 0 012
0 40	0 222 ± 0 008	0 248 ± 0 020	0 218 ± 0 045
0 6	0 309 ± 0 017	0 322 ± 0 017	0 256 ± 0 015
0 8	0 351 ± 0 025	0 362 ± 0 011	0 324 ± 0 021
1 0	0 448 ± 0 300	0 433 ± 0 025	----------
2 0	1 003 ± 0 039	1 030 ± 0 059	0 947 ± 0 046

3.2.2.3 Effect of insulin on CHOK1 cells in SFM

The response to bovine insulin was investigated in the serum-free system The results are shown in Figure 3 2 2 3 For Assays 1, 2 and 4, there was an almost constant stimulation from 0 1 - 0 5μg/ml to 1 - 2 5μg/ml The extent of stimulation decreased at 5 - 10μg/ml and increased slightly again at 20μg/ml In assays 1 and 2, the stimulation at 0 1μg/ml was almost as good as that seen at 1 to 2 5μg/ml, for assays 3 and 4, the extent of stimulation was much greater

3.2.2.4 Effect of transferrin on growth of CHOK1 cells in SFM

The response of CHOK1 cells to bovine transferrin was investigated in SFM Mendiaz suggested that transferrin was not necessary if Fe_2SO_4 were present, but it was incorporated both into the SFM The results are shown in Figure 3 2 2 4

Little stimulation in comparison to that elicited by insulin, was seen in assays 1 and 2, while slight inhibition was seen in assays 3 and 4 Assays were set up with Fe_2SO_4 in the SFM This was done in order to see if transferrin was required in addition to Fe_2SO_4 From these results it would appear that transferrin need not be added to the serum-free medium

3.2.2.5 Effect of albumin on CHOK1 cells in SFM

The effects of BSA fraction V and fatty acid free are shown in Figure 3 2 2 5 The results obtained are similar to those observed for MDCK cells in SFM Stimulation was seen at the lowest concentration of both albumins but as the concentration increased, the effects became rapidly inhibitory BSA fatty acid free was marginally better than BSA fraction V while stimulating CHOK1 cells at 0 1mg/ml At higher concentrations the fatty acid free was less inhibitory than the fraction V

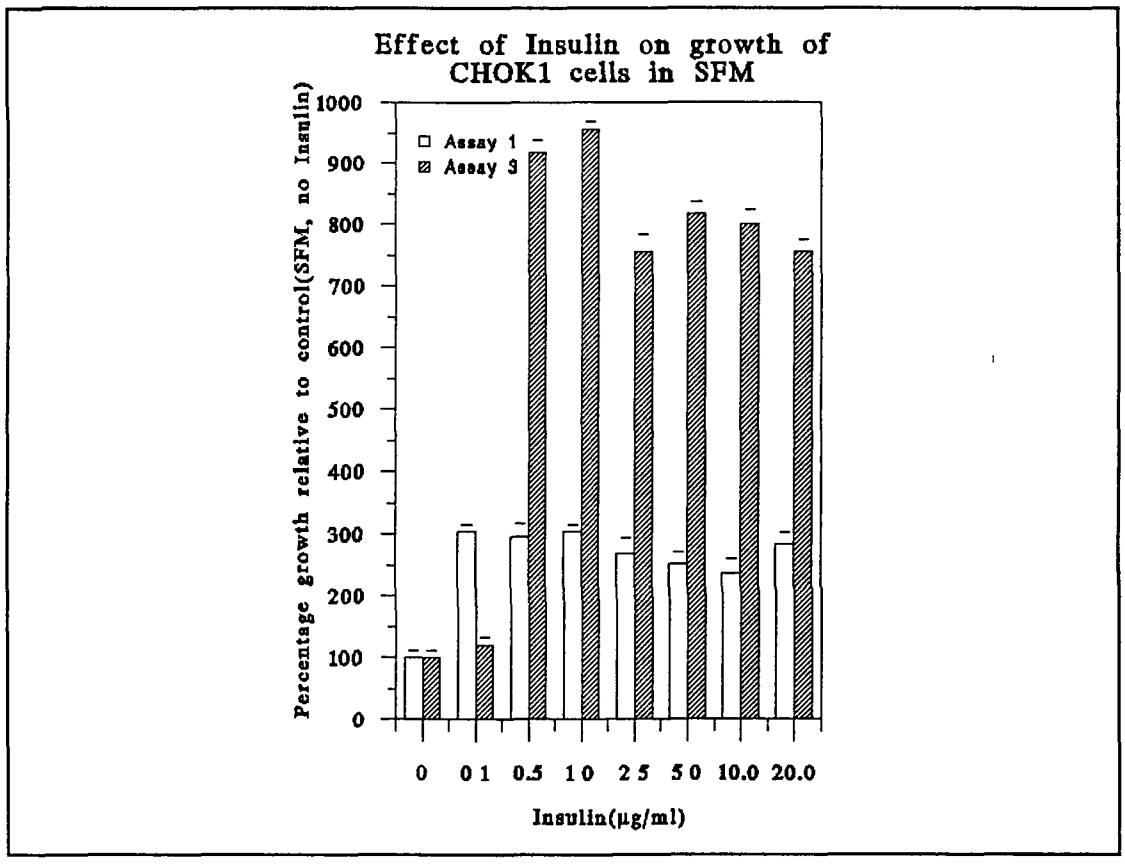


Figure 3.2.2.3 shows the effect of insulin on CHOK1 cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments The results of 4 separate experiments are shown in Table 3 2 2 3

Table 3.2.2.3 Effect of insulin (µg/ml) on CHOK1 cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 11 5	100 0 ± 13 1	100 0 ± 20 9	100 0 ± 18 0
0 1µg/ml	304 3 ± 11 9	518 4 ± 36 0	120 9 ± 20 4	118 5 ± 14 8
0 5µg/ml	296 5 ± 21 6	440 1 ± 18 4	917 4 ± 69 6	740 7 ± 74 8
1 0µg/ml	303 8 ± 12 9	406 5 ± 16 4	956 0 ± 113	644 4 ± 40 0
2 5µg/ml	269 0 ± 26 0	369 1 ± 29 6	756 5 ± 65 2	622 2 ± 55 5
5 0µg/ml	251 0 ± 20 2	330 1 ± 32 8	817 4 ± 60 9	595 2 ± 85 2
10 0µg/ml	235 6 ± 24 3	286 0 ± 18 7	800 0 ± 45 2	540 7 ± 100
20 0µg/ml	284 0 ± 18 2	390 2 ± 26 6	756 5 ± 73 9	700 0 ± 77 7

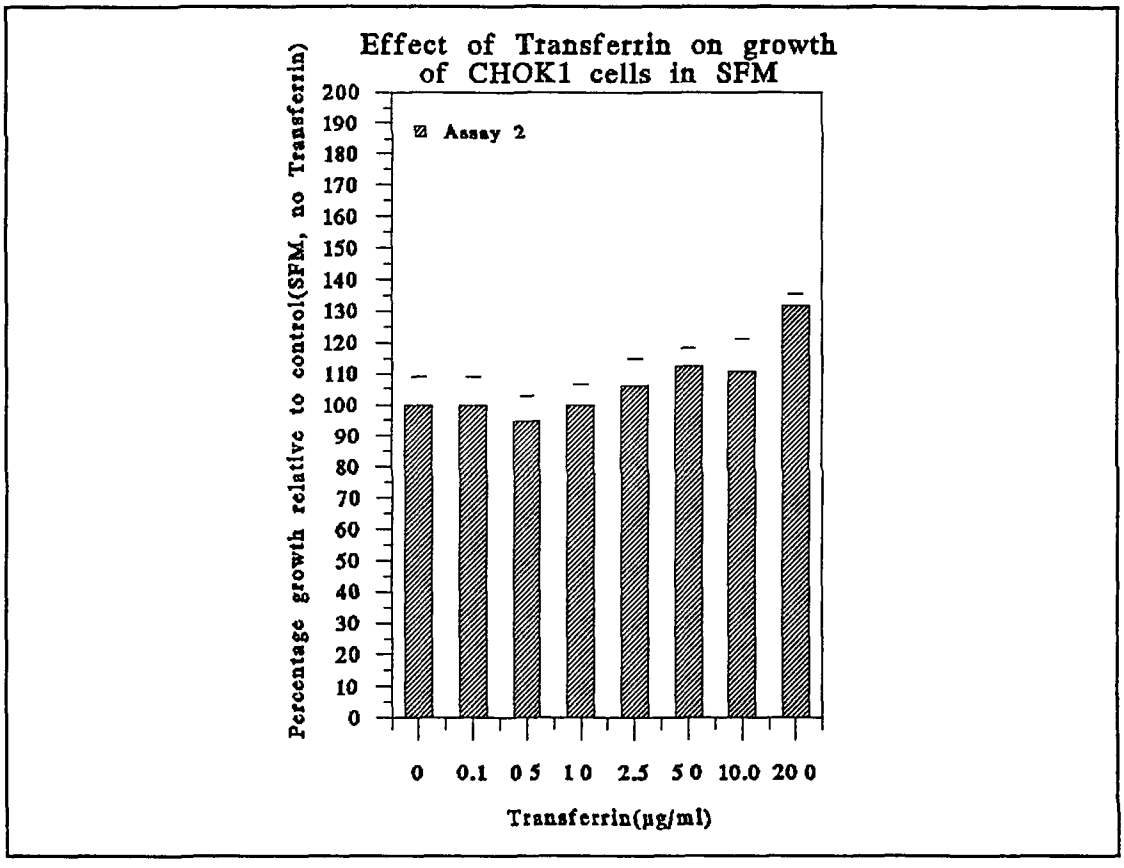


Figure 3.2.2.4 shows the effect of transferrin on CHOK1 cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments The results for 4 separate assays are shown in Table 3 2 2 4

Table 3.2.2.4 Effect of transferrin (µg/ml) on CHOK1 cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 8 69	100 0 ± 8 85	100 0 ± 7 48	100 0 ± 5 01
0 1µg/ml	102 3 ± 8 39	99 71 ± 9 28	91 76 ± 8 98	86 74 ± 6 06
0 5µg/ml	94 04 ± 5 80	94 67 ± 8 03	94 37 ± 7 86	94 32 ± 5 01
1 0µg/ml	91 75 ± 8 25	99 58 ± 7 06	92 88 ± 8 61	87 88 ± 7 58
2 5µg/ml	88 14 ± 5 34	106 1 ± 8 78	91 01 ± 7 34	87 12 ± 6 44
5 0µg/ml	92 89 ± 8 88	112 2 ± 6 00	88 76 ± 2 99	84 85 ± 6 82
10 0µg/ml	120 6 ± 4 92	110 7 ± 10 5	85 77 ± 8 99	87 88 ± 8 71
20 0µg/ml	160 9 ± 12 9	131 5 ± 3 83	91 76 ± 5 99	98 86 ± 9 47

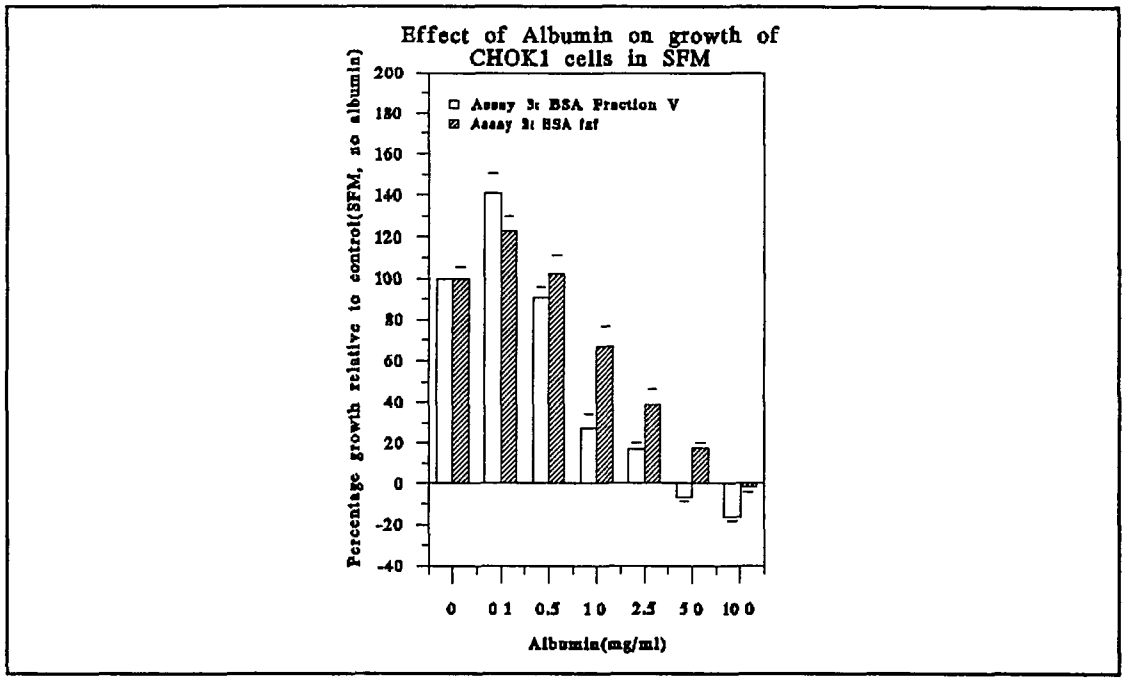


Figure 3.2.2.5 shows the effect of BSA fraction V and fatty acid free on CHOK1 cells in serum-free medium Results are expressed as the average percentage growth relative to control (SFM, no BSA) ± standard deviation (n=8) Abbreviations Con = Control (no BSA) Acid phosphatase was used as the end point for experiments

Table 3.2.2.5a Effect of BSA fraction V (mg/ml) on CHOK1 cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 8 50	100 0 ± 7 02	100 0 ± 8 80	100 0 ± 6 56
0 1mg/ml	104 8 ± 15 1	160 0 ± 13 7	140 7 ± 9 55	128 3 ± 11 8
0 5mg/ml	51 60 ± 10 1	110 0 ± 17 4	90 95 ± 5 02	82 03 ± 7 35
1 0mg/ml	42 95 ± 6 61	48 15 ± 7 66	27 13 ± 7 44	51 47 ± 4 98
2 5mg/ml	28 32 ± 1 57	16 56 ± 0 60	17 08 ± 3 01	11 30 ± 4 50
5 0mg/ml	6 13 ± 2 20	4 85 ± 3 50	-6 53 ± 2 01	-10 2 ± 8 17
10 0mg/ml	-0 94 ± 0 47	-8 91 ± 9 20	-16 1 ± 2 01	-18 4 ± 6 54

Table 3.2 2.5b Effect of BSA fatty acid free (mg/ml) on CHOK1 cells in SFM

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
0 0	100 0 ± 24 3	100 0 ± 8 80	100 0 ± 5 80	100 0 ± 7 49
0 1mg/ml	128 8 ± 29 2	173 2 ± 11 0	122 6 ± 7 24	115 8 ± 11 5
0 5mg/ml	37 94 ± 3 36	-2 70 ± 0 18	102 3 ± 9 00	79 84 ± 7 90
1 0mg/ml	24 67 ± 8 60	-3 40 ± 0 44	66 97 ± 9 95	52 96 ± 1 58
2 5mg/ml	13 76 ± 1 12	3 66 ± 0 59	38 98 ± 7 69	25 29 ± 2 77
5 0mg/ml	6 730 ± 1 49	3 76 ± 0 40	17 64 ± 2 71	4 26 ± 2 60
10 0mg/ml	-3 60 ± 5 61	-3 70 ± 0 51	-1 81 ± 2 71	-9 88 ± 1 58

3.2.2.6 Subculture of CHOK1 cells in serum-free medium

An experiment to investigate the ability of CHOK1 cells to grow in serum-free medium over a period of subcultures was carried out The results are shown in Table 3 2 2 6 1

Table 3.2.2.6.1 Subculture of CHOK1 cells in SFM (Cell Yield x 10^6/flask)

Passage	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
1	2 16 ± 0 45	2 66 ± 0 33	1 71 ± 0 57
2	1 99 ± 0 28	1 17 ± 0 07	0 91 ± 0 09
3	0 748 ± 0 16	1 85 ± 0 63	1 58 ± 0 54
4	0 325 ± 0 09	0 363 ± 0 15	0 31 ± 0 06

Results expressed as average cell yield ± standard deviation (n=3)

In the first three subcultures the cells grew quite well It was noted though, that a higher centrifugal speed or a longer centrifuging time was required to get the cells to form a pellet On the fourth subculture, there was a total fall off in growth When the cells were reset up, very few cells attached After 15 days in the fourth subculture, there had been no increase in the amount of cells attached to the surface

It may be that the trace elements originally left out of the medium play a vital role and their importance is not seen due to the presence of material carried over from the serum It may also be related to the use of attachment factors These cells do grow in suspension under certain conditions and these conditions may have existed in the fourth subculture In order to see if the trace elements were responsible for the SFM being unable to support the growth of CHOK1 cells beyond four passages, a growth curve was set up to compare the SFM with and without trace elements The results are shown in Table 3 2 2 6 2

Table 3.2.2 6.2 Growth curve for CHOK1 cells in SFM and SSM (Cell yield x 10^{-4} cells/well)

Day	SFM	SFM + TE	SSM
3	3 797 ± 0 37	4 508 ± 0 36	11 85 ± 1 36
5	24 28 ± 3 09	27 66 ± 2 65	88 35 ± 3 68
6	64 47 ± 4 64	79 46 ± 2 76	140 9 ± 9 16
7	99 36 ± 4 65	101 5 ± 5 15	156 0 ± 12 6
8	72 06 ± 20 0	123 6 ± 2 46	113 2 ± 12 3

Results expressed as average cell yield ± standard deviation (n=3) Abbreviations TE = trace elements as Appendix J, SSM = serum-supplemented medium (5% FCS)

These results show that the addition of trace elements resulted in slightly better growth than the SFM alone. Growth in comparison to the serum control appears better than that obtained in section 3.2.2.1. When the CHOK1 cells were subcultured in the SFM with trace elements, the cells grew for up to 5 passages before dying away (Table 3.2.2.6.3). In this subculture experiment, cells in the SFM medium without trace elements only grew for 3 passages. No attachment was seen in the fourth passage or in passage 6 for the cells in the SFM with the trace elements. In the subcultures, an overall increase in growth was observed in the second last passage. When the cells were passaged into a new flask, growth was very low or totally stopped (it appeared as if the cells did not attach to the flask). In a subsequent subculture experiment (Appendix C), growth was maintained for 9 subcultures before cells stopped growing. Why there was a sudden increase in growth in the second last passage is unknown. It may be that the cells used up some cellularly stored component which was necessary for growth. The lack of growth regulating factors may also have made the cells more susceptible to cell death.

Table 3.2.2.6.3 Subculture for CHOK1 cells in SFM (Cell yield x 10^{-6})

SERUM-FREE MEDIUM WITHOUT TE			
PASSAGE	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
1	1.687 $\pm$ 0.03	1.415 $\pm$ 0.02	1.220 $\pm$ 0.003
2	1.210 $\pm$ 0.15	1.410 $\pm$ 0.03	1.378 $\pm$ 0.04
3	3.930 $\pm$ 2.26	3.747 $\pm$ 0.09	3.835 $\pm$ 0.09
SERUM-FREE MEDIUM WITH TE			
PASSAGE	EXPERIMENT 1	EXPERIMENT 2	EXPERIMENT 3
1	2.092 $\pm$ 0.04	1.305 $\pm$ 0.21	1.475 $\pm$ 0.07
2	1.833 $\pm$ 0.03	2.012 $\pm$ 0.04	1.645 $\pm$ 0.25
3	1.825 $\pm$ 0.22	1.930 $\pm$ 0.03	1.905 $\pm$ 0.20
4	0.907 $\pm$ 0.03	0.577 $\pm$ 0.06	0.532 $\pm$ 0.08
5	0.493 $\pm$ 0.05	1.650 $\pm$ 0.01	1.005 $\pm$ 0.03

Results expressed as average cell yield $\pm$ standard deviation (n=3). Abbreviations: TE = trace elements as Appendix J.

In summary, for CHOK1 cells the SFM designed by Mendiaz was able to support growth, but not always over long periods of time (the longest subculture was over 9 passages and 36 days). Acid phosphatase production showed a linear relationship to cell number and so was used to see how responsive the CHOK1 cells were to insulin, transferrin and albumin. Insulin showed

increasing stimulation with increasing concentration Transferrin elicited variable results but the extent of which was negligible when the errors were taken into account Albumin, either BSA fraction V or fatty acid free, showed little or no effect at low concentrations and increasing inhibition at higher concentrations Thus we had a system in which the cells could be subcultured for one passage before assaying the effects of insulin and transferrin and studying the possible replacements of these serum-derived proteins

3.3 REPLACEMENT OF TRANSFERRIN IN SFM FOR MDCK CELLS

Early reports showed the that diferric transferrin was necessary for growth of many cells under serum-free conditions (Barnes and Sato, 1980(a,b), Laskey *et al* 1988, Hashizuma *et al* 1983) In later studies, transferrin was replaced by a wide range of compounds including simple soluble iron salts *e g* Fe_2SO_4 (Mendiaz *et al* 1986) and ferric citrate (Amouric *et al* 1984), insoluble iron *e g* ferric hydroxide (Kovar, 1990), and complex iron chelators *e g* ferric salicylaldehyde and ferric pyridoxal isonicotinoyl hydrazone (Laskey *et al* 1988, Landschulz *et al* , 1984)

Transferrin can carry out two functions in a serum-free medium Predominately it acts as a source and carrier of iron It may also act as a detoxifying agent, by inhibiting lipid peroxidation (Gutteridge, 1981) or by chelating other metals Iron can be transported into the cell in one of two ways, *via* a specific mechanism which involves endocytosis of transferrin - transferrin receptor complexes, or *via* non transferrin-receptor-specific means (Richardson and Baker, 1992, Kaplan, 1991)

For MDCK cells, transferrin and PGE_1 were found to be the most important components of the serum-free medium designed by Taub *et al* , (1979) The relationship between apo- and diferric-transferrin was investigated for MDCK cells in iron poor medium by Eby *et al* (1992) They found that diferric-transferrin was able to support growth at concentrations up to 50 - 100μg/ml, while the apo-transferrin was inactive over the whole concentration range tested (0 05 - 100μg/ml)

In this section a comparison of apo-, partially- and fully-saturated bovine transferrin was made, to see how the quantity of iron bound to transferrin affected its activity In the following section, a variety of iron containing complexes were investigated in serum-free medium to see if any or all of these complexes could replace transferrin On the basis of these results, selected complexes were tested over an extended period of time to see if the activity seen in a once off experiment could be maintained through several subcultures in serum-free medium

3.3.1.1 Apo-transferrin

The results for apo-transferrin are shown in Figure 3 3 1 1 At low concentrations of apo-transferrin (0 05 to 0 5μg/ml), no stimulation was seen in two of three separate assays with 30% inhibition relative to the control (no transferrin) seen in the third At higher concentrations, the transferrin became stimulatory, reaching maximum stimulation at 5 to 10μg/ml (Note the concentration of Fe_2SO_4 in the basal medium was 0 417μg/ml according to the Sigma catalogue) The extent of maximum stimulation varied from assay to assay (4 3-fold to 12-fold over the control) Apo-transferrin was as stimulatory as the control transferrin (partially-saturated) at 5μg/ml Thereafter, the extent of activity decreased at 50μg/ml

3.3.1.2 Partially-saturated Transferrin

The results for the effect of partially-saturated transferrin are shown in Figure 3 3 1 2 At the lowest concentrations tested (0 05 - 0 1μg/ml), inhibition of up to 24% was seen The transferrin then became stimulatory at 0 5 - 1μg/ml and continued to stimulate growth in a concentration-dependent manner reaching maximum stimulation at 10μg/ml At 50μg/ml, there was a loss of stimulation with inhibition seen in two of the three assays The serum-free control contained 5μg/ml of partially-saturated transferrin and the activity in the assays at 5μg/ml showed similar stimulation to the Tf control

3.3.1.3 Fully-saturated Transferrin

At the lowest concentrations of fully-saturated transferrin tested (0 05 - 0 5μg/ml), inhibition was seen (Figure 3 3 1 3) Thereafter, stimulation occurred and increased sharply between 1 and 5μg/ml After this, the increase in stimulation was more gradual, reaching maximum stimulation at the highest concentration tested (50μg/ml) At 5μg/ml, fully-saturated transferrin was less stimulatory than the partially-saturated transferrin at 5μg/ml Maximum stimulation of up to and over 5-fold over the control was achieved The extent of stimulation varied from assay to assay

The results show that all of the transferrins were equally stimulatory at concentrations of 5 - 10μg/ml At higher concentrations of 50μg/ml, the apo- and partially-saturated transferrin became less stimulatory or inhibitory, while the fully-saturated transferrin continued to be stimulatory

The extent of stimulation by the fully-saturated transferrin compared favourably with the diferric form used by Eby *et al* , (1992) In the case of apo-transferrin and partially-saturated

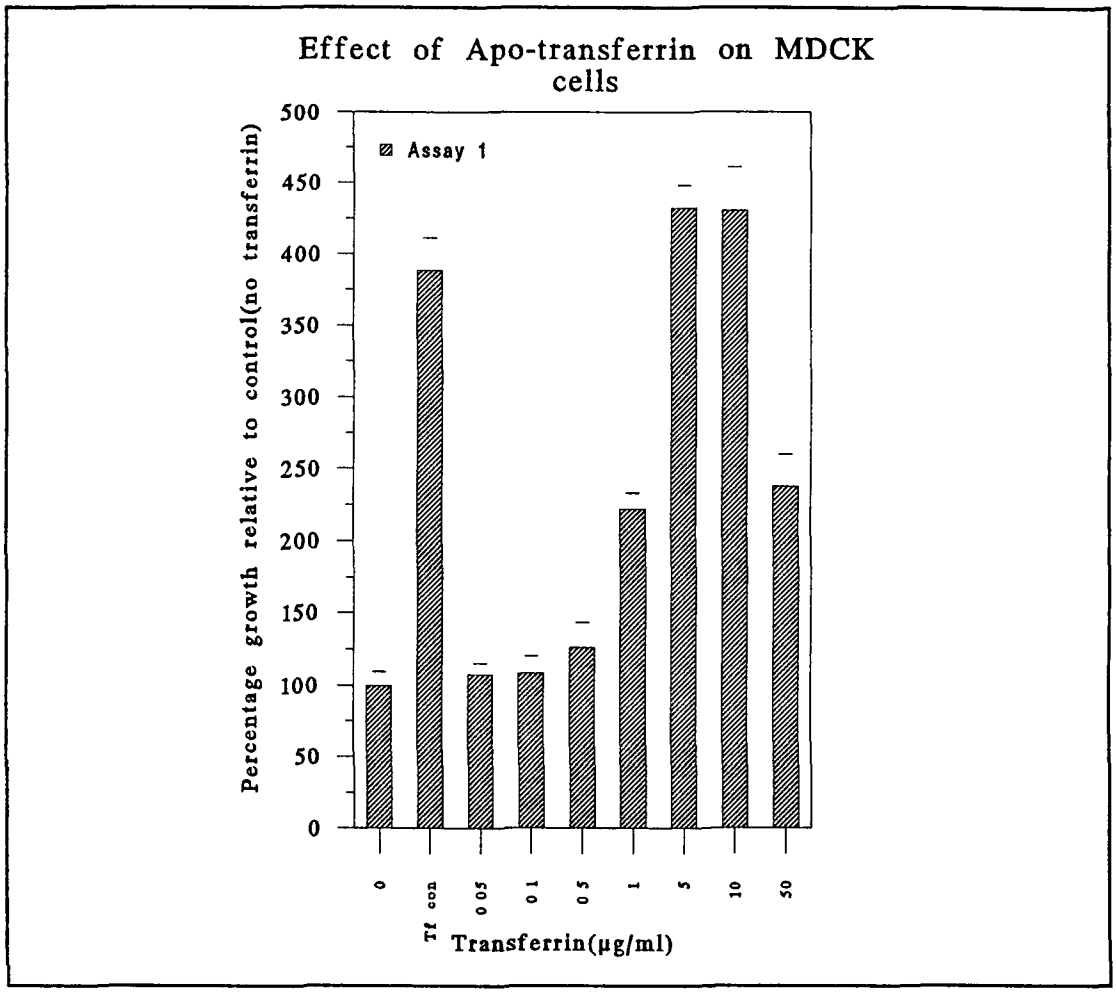


Figure 3.3.1.1 shows the growth response of MDCK cells to bovine apo-transferrin (iron free) under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3 1 1 Tf con refers to the recommended concentration of 5µg/ml partially saturated transferrin

Table 3.3.1.1 Growth response to apo-transferrin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 ± 9 23	100 0 ± 9 41	100 0 ± 14 8
Tf con	388 6 ± 22 9	918 0 ± 66 2	233 3 ± 16 0
+ 0 05 µg/ml	107 7 ± 7 90	73 92 ± 6 23	126 4 ± 13 0
+ 0 1 µg/ml	108 7 ± 11 8	69 13 ± 8 22	106 7 ± 21 3
+ 0 5 µg/ml	126 5 ± 16 6	93 29 ± 5 35	231 8 ± 39 8
+ 1 0 µg/ml	222 3 ± 11 1	208 7 ± 20 7	342 4 ± 35 0
+ 5 0 µg/ml	432 2 ± 16 0	1124 ± 108	449 6 ± 37 8
+ 10 0 µg/ml	430 4 ± 30 4	1210 ± 109	248 1 ± 33 8
+ 50 0 µg/ml	237 9 ± 21 7	1097 ± 88 6	242 8 ± 19 6

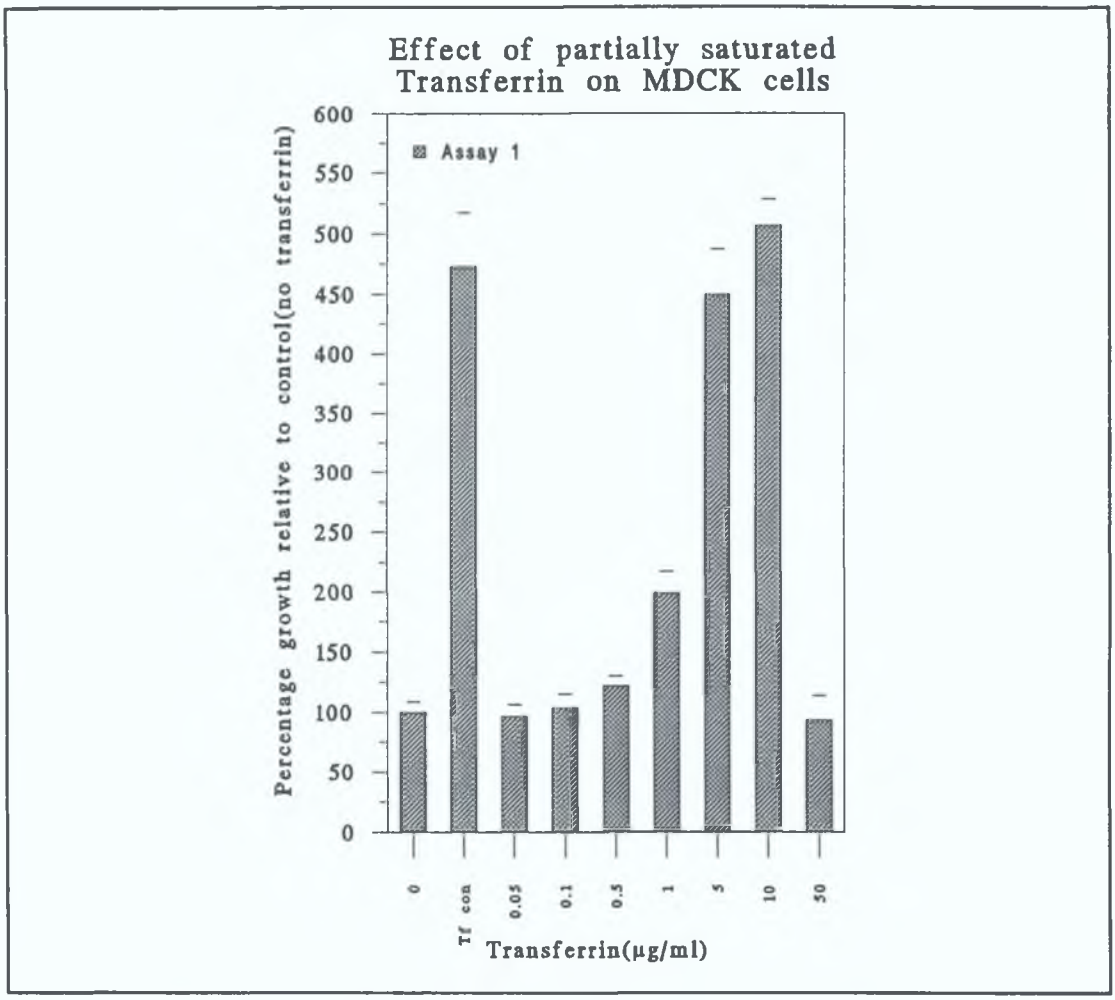


Figure 3.3.1.2 shows the growth response of MDCK cells to partially-saturated bovine transferrin under serum-free conditions. The results are expressed as the average percentage growth relative to control (no transferrin) $\pm$ standard deviation (n=8). Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3.3.1.2. Tf con refers to the recommended concentration of 5μg/ml partially-saturated transferrin.

Table 3.3.1.2 Growth response to partially-saturated transferrin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0.0 μg/ml	100.0 $\pm$ 8.49	100.0 $\pm$ 8.86	100.0 $\pm$ 8.25
Tf con	473.0 $\pm$ 44.2	318.3 $\pm$ 38.9	448.9 $\pm$ 21.9
+ 0.05 μg/ml	96.43 $\pm$ 9.88	76.28 $\pm$ 6.74	94.33 $\pm$ 7.80
+ 0.1 μg/ml	103.8 $\pm$ 11.5	72.04 $\pm$ 4.67	104.2 $\pm$ 7.09
+ 0.5 μg/ml	121.1 $\pm$ 8.49	95.08 $\pm$ 6.79	143.3 $\pm$ 7.09
+ 1.0 μg/ml	199.0 $\pm$ 18.3	179.5 $\pm$ 18.3	220.2 $\pm$ 25.2
+ 5.0 μg/ml	449.5 $\pm$ 37.7	344.3 $\pm$ 32.2	555.3 $\pm$ 43.3
+ 10.0 μg/ml	506.7 $\pm$ 21.1	398.1 $\pm$ 30.5	586.5 $\pm$ 38.3
+ 50.0 μg/ml	92.94 $\pm$ 20.7	242.5 $\pm$ 41.4	45.63 $\pm$ 4.72

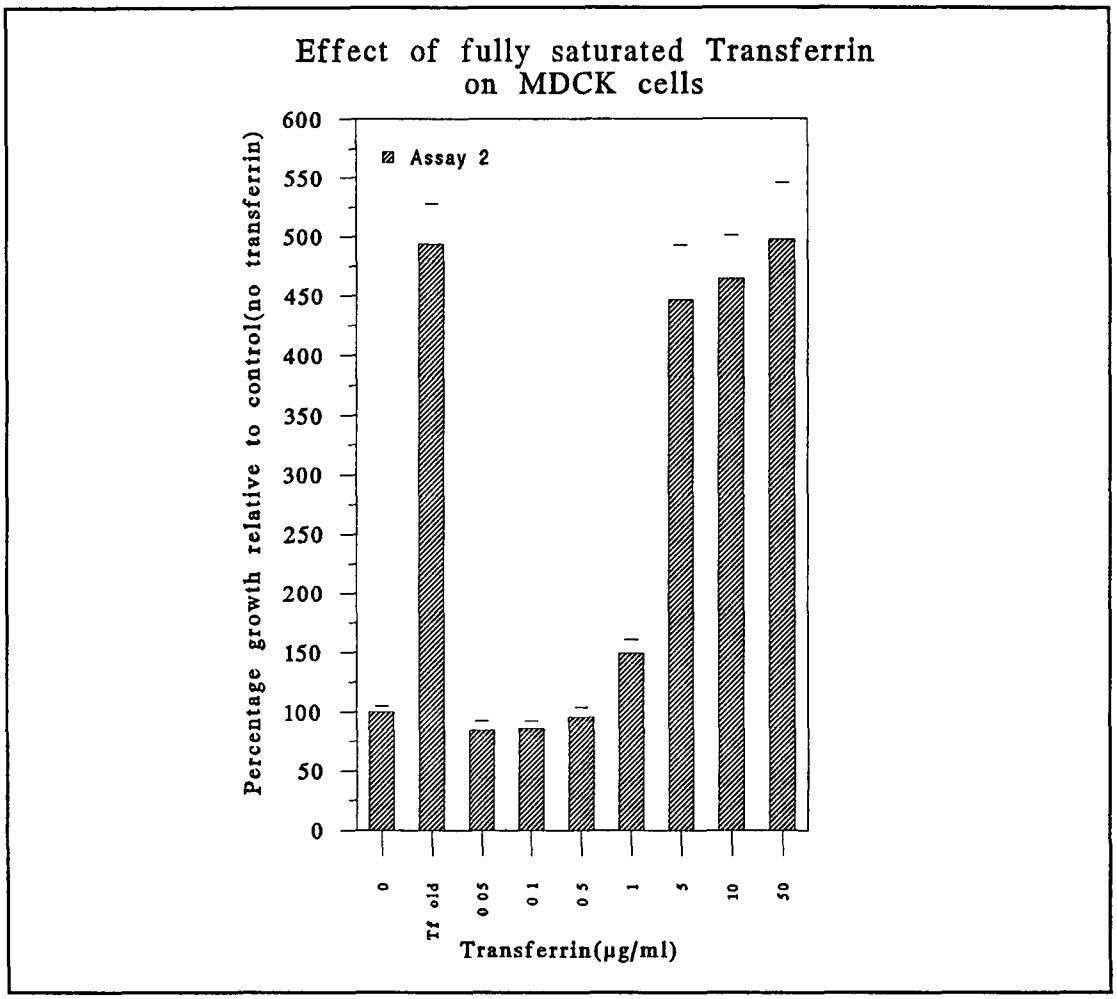


Figure 3.3.1.3 shows the growth response of MDCK cells to fully-saturated bovine transferrin under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3 1 3 Tf con refers to the recommended concentration of 5μg/ml partially-saturated transferrin

Table 3.3.1.3 Growth response to fully-saturated transferrin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 $\pm$ 4 78	100 0 $\pm$ 5 30	100 0 $\pm$ 5 47
Tf con	418 8 $\pm$ 23 1	493 8 $\pm$ 34 6	356 6 $\pm$ 37 6
+ 0 05 µg/ml	92 47 $\pm$ 8 06	84 77 $\pm$ 7 56	73 96 $\pm$ 4 73
+ 0 1 µg/ml	93 00 $\pm$ 5 38	86 42 $\pm$ 6 17	78 69 $\pm$ 5 32
+ 0 5 µg/ml	118 3 $\pm$ 7 53	95 55 $\pm$ 8 31	87 57 $\pm$ 8 28
+ 1 0 µg/ml	155 1 $\pm$ 4 15	149 4 $\pm$ 11 1	128 4 $\pm$ 6 51
+ 5 0 µg/ml	425 8 $\pm$ 26 3	446 9 $\pm$ 45 7	281 7 $\pm$ 21 3
+ 10 0 µg/ml	459 7 $\pm$ 32 3	464 4 $\pm$ 36 8	350 0 $\pm$ 33 7
+ 50 0 µg/ml	527 9 $\pm$ 25 3	497 5 $\pm$ 48 1	370 0 $\pm$ 28 9

transferrin, the inhibition seen at higher concentrations may have been due to the two species removing iron from the medium which would under normal circumstances have entered by transferrin-mdependent means and so making iron less available to the cells It was also possible that the tranferrin at high concentrations had some other adverse effects on cell growth *e g* binding other metals required for growth

It was evident from these results that up to a concentration of 5-10μg/ml, the apo- and partially-saturated transferrins were as active as the fully-saturated transferrin This indicated that regardless of the level of iron saturation, transferrin was chelating iron from the medium and delivering it to the cells It was of interest, therefore, to see how MDCK cells responded to a variety of iron containing complexes

3.3.2 ALTERNATIVES TO TRANSFERRIN

In the previous section, the importance of transferrin to MDCK cells was shown All transferrins, regardless of the level of iron saturation were able to stimulate growth to a similar extent in the range 5 - 10μg/ml This stimulation may have been due to a combination of iron delivery and detoxification It was therefore of interest to see if simple ferric salts or ferric complexes could stimulate such activity The following factors were chosen sodium nitroprusside, ferric nitrate, iron choline citrate, ferric citrate, ferric ammonium citrate, ferric ammonium sulphate and ferrous sulphate

3.3.2.1 **Sodium nitroprusside** (SNP)

The effect of SNP on the growth of MDCK cells is shown in Figure 3 3 2 1 Some inhibition at 0 05μg/ml was seen but at higher concentrations, stimulation occurred and increased with increasing concentration to reach a maximum stimulation of 3-fold to 5-fold over the control at 1μg/ml Between 1 - 10μg/ml the stimulation decreased By 50μg/ml, inhibition of 40 - 70% was seen depending on the assay Growth at 1μg/ml was not quite as good as that seen with the Tf control containing 5μg/ml partially-saturated transferrin The ratio of highest stimulation to the Tf control was 1 29, 0 56 and 0 856 for the three independent assays (average $\pm$ standard deviation of 0 902 $\pm$ 0 367)

3.3.2.2 **Ferric Nitrate** (FN)

No inhibition was seen at lower concentrations (Figure 3 3 2 2) Maximum stimulation was seen at 0 5μg/ml with a 2 6-fold to 3-fold stimulation over the control (no transferrin) By 5μg/ml, the ferric nitrate had become inhibitory and increasing the concentration resulted in increased inhibition, with a maximum inhibition of 60 - 90% seen at 50μg/ml The ratio of highest stimulation to the Tf control was 0 752, 0 585 and 0 634 for the three independent assays (average $\pm$ standard deviation of 0 657 $\pm$ 0 0858)

3.3.2.3 **Iron choline citrate** (ICC)

At the lowest concentrations tested (0 05 - 0 1μg/ml), some slight inhibition was seen (Figure 3 3 2 3) From 0 1μg/ml stimulation occurred, reaching a maximum at 1 0μg/ml and then decreasing and increasing again in the range 5 - 50μg/ml At 1μg/ml, ICC was almost as good as the Tf control The ratio of highest stimulation to the Tf control was 0 962, 0 93 and 0 762 for the three independent assays (average $\pm$ standard deviation of 0 885 $\pm$ 0 107)

3.3.2.4 Ferric Citrate (FC)

The effect of ferric citrate is shown in Figure 3 3 2 4 On addition of 0 05μg/ml FC, a 2-fold increase in growth was observed A similarly high stimulation at the lowest concentration was seen with FAS, FN and Fe_2SO_4 Stimulation increased gradually reaching a maximum of 2 7-fold to 3 1-fold over the control at 0 1 - 1 0μg/ml At higher concentrations (5 - 50μg/ml), FC became inhibitory with up to 80% inhibition seen in two of the three assays at 50μg/ml In comparison to the Tf control, maximum growth with FC was 60 - 80% of that achieved by the Tf control (ratios of highest stimulation of FC to Tf control were 0 604, 0 591 and 0 82 average $\pm$ standard deviation of 0 672 $\pm$ 0 129)

3.3.2.5 Ferric Ammonium Citrate (FAC)

FAC showed little stimulation at 0 05 - 0 1μg/ml (Figure 3 3 2 5) At 0 5μg/ml a 3-fold to 4-fold increase in growth was seen over the control At higher concentrations the extent of stimulation tailed off, with the exception of a slight increase occurring between 10 and 50μg/ml The ratio of highest stimulation to the Tf control was 0 864, 0 547 and 0 607 for the three independent assays (average $\pm$ standard deviation of 0 673 $\pm$ 0 168)

3.3.2.6 Ferric Ammonium Sulphate (FAS)

The effect of FAS on the growth of MDCK cells is shown in Figure 3 3 2 6 No inhibition was seen at the lowest concentration tested (0 05μg/ml) Increasing the concentration gave increasing growth with a maximum stimulation of 3-fold to 4-fold over the control (without transferrin) at 1μg/ml Abruptly at 5μg/ml, FAS became inhibitory, with 75 - 87% inhibition occurring at 50μg/ml In comparison to the Tf control, growth was almost as good as with 5μg/ml partially-saturated transferrin (the ratios of maximum stimulation to Tf control were 0 954, 0 904 and 0 807 average $\pm$ standard deviation of 0 888 $\pm$ 0 075)

3.3.2.7 Ferrous Sulfate (Fe_2SO_4)

The effect of Fe_2SO_4 on the growth of MDCK cells is shown in Figure 3 3 2 7 At the lowest concentration tested (0 05μg/ml), a 2-fold stimulation over the control (without transferrin) was seen Stimulation increased with increasing concentration, up to a maximum reached at 0 5μg/ml At 5μg/ml, Fe_2SO_4 became inhibitory, with a maximum inhibition of 87 - 96% at 50μg/ml The ratio of maximum stimulation to the Tf control was 1 387, 0 810 and 0 952 for the three assays, showing it to be almost as good as transferrin (average $\pm$ standard deviation of 1 049 $\pm$ 0 301)

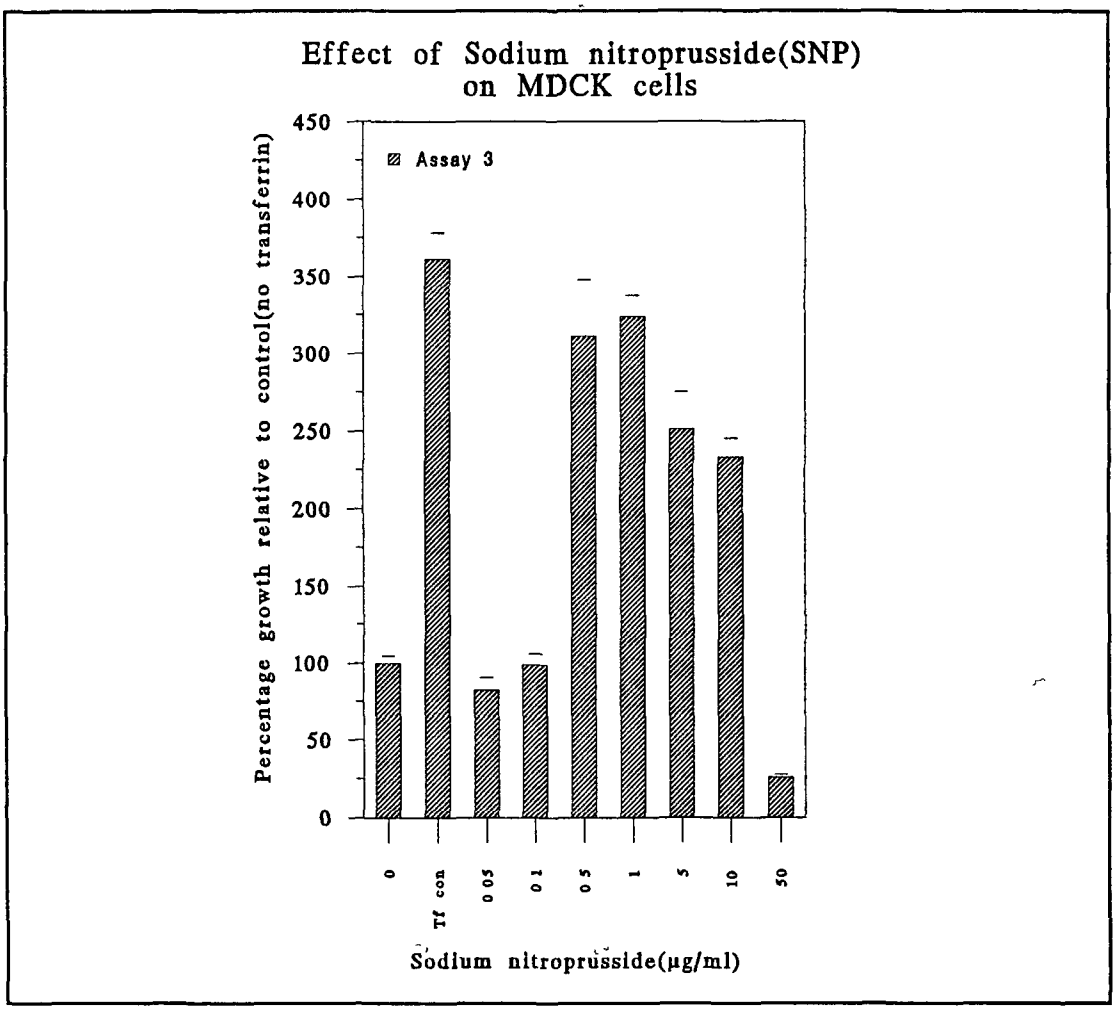


Figure 3.3.2.1 shows the growth response of MDCK cells to sodium nitroprusside (SNP) under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3 2 1 Tf con refers to the recommended concentration of 5µg/ml partially-saturated transferrin

Table 3.3.2.1 Growth response to SNP

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 ± 8 30	100 0 ± 6 80	100 0 ± 4 55
Tf con	407 9 ± 31 9	426 6 ± 35 1	361 7 ± 16 5
+ 0 05 µg/ml	114 8 ± 5 56	91 49 ± 7 44	82 62 ± 8 03
+ 0 1 µg/ml	129 1 ± 7 13	104 2 ± 11 7	94 14 ± 6 84
+ 0 5 µg/ml	351 6 ± 34 4	262 8 ± 23 4	311 1 ± 36 7
+ 1 0 µg/ml	497 4 ± 29 1	282 9 ± 26 6	324 0 ± 13 7
+ 5 0 µg/ml	332 2 ± 16 9	170 9 ± 19 9	251 3 ± 23 9
+ 10 0 µg/ml	224 9 ± 21 9	150 9 ± 5 60	233 0 ± 12 1
+ 50 0 µg/ml	57 82 ± 2 52	32 34 ± 2 23	26 07 ± 1 50

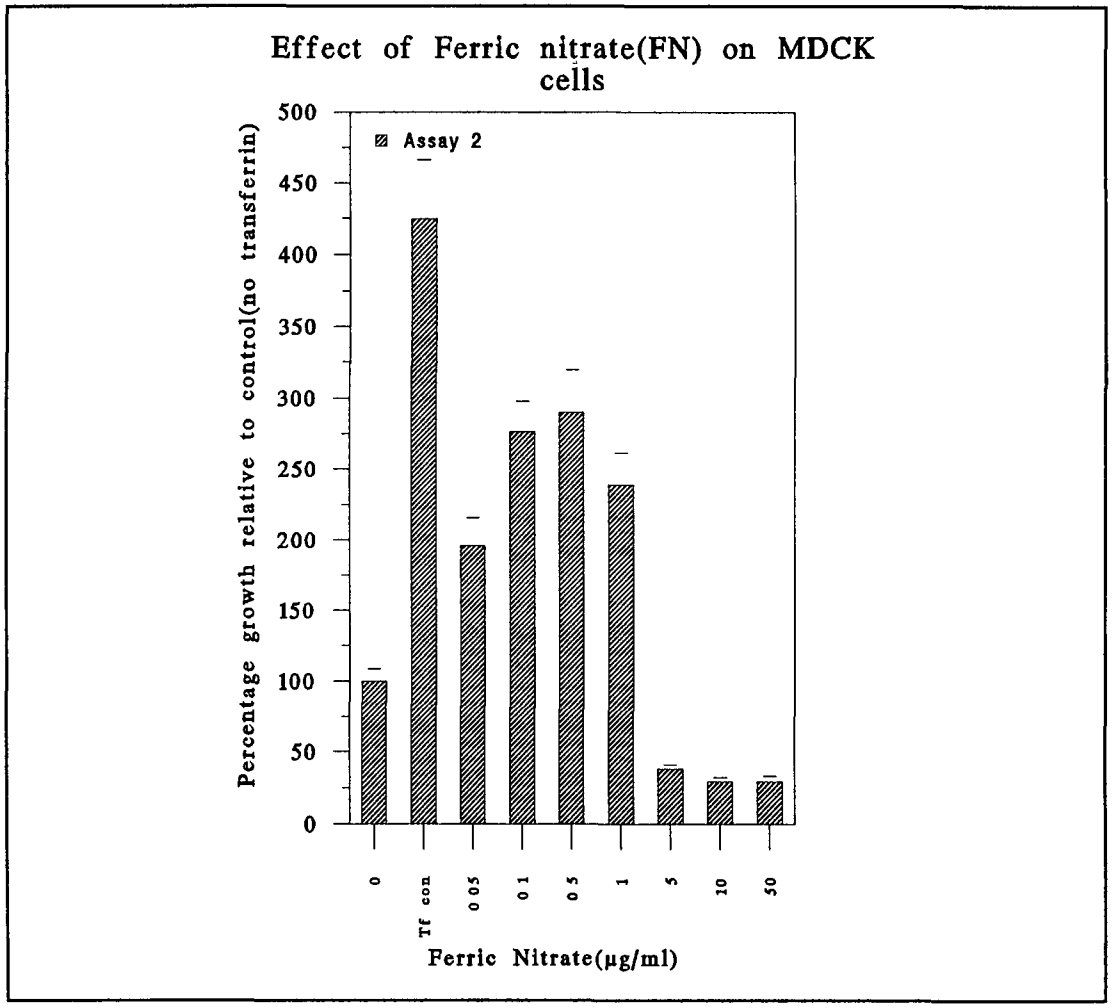


Figure 3.3.2.2 shows the growth response of MDCK cells to ferric nitrate (FN) under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3 2 2 Tf con refers to the recommended concentration of 5µg/ml partially-saturated transferrin

Table 3.3.2.2 Growth response to FN

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 ± 7 71	100 0 ± 8 73	100 0 ± 8 33
Tf con	371 6 ± 41 3	424 7 ± 41 6	358 2 ± 32 8
+ 0 05 µg/ml	162 9 ± 18 8	196 0 ± 19 8	192 7 ± 12 7
+ 0 1 µg/ml	171 3 ± 10 3	276 0 ± 21 8	250 4 ± 31 3
+ 0 5 µg/ml	304 4 ± 22 8	290 0 ± 29 7	263 8 ± 31 6
+ 1 0 µg/ml	223 3 ± 14 3	238 6 ± 22 8	184 9 ± 40 9
+ 5 0 µg/ml	79 04 ± 8 53	38 12 ± 2 78	18 95 ± 4 17
+ 10 0 µg/ml	50 73 ± 6 72	29 20 ± 2 99	13 43 ± 2 74
+ 50 0 µg/ml	40 44 ± 2 94	29 10 ± 4 15	10 32 ± 1 45

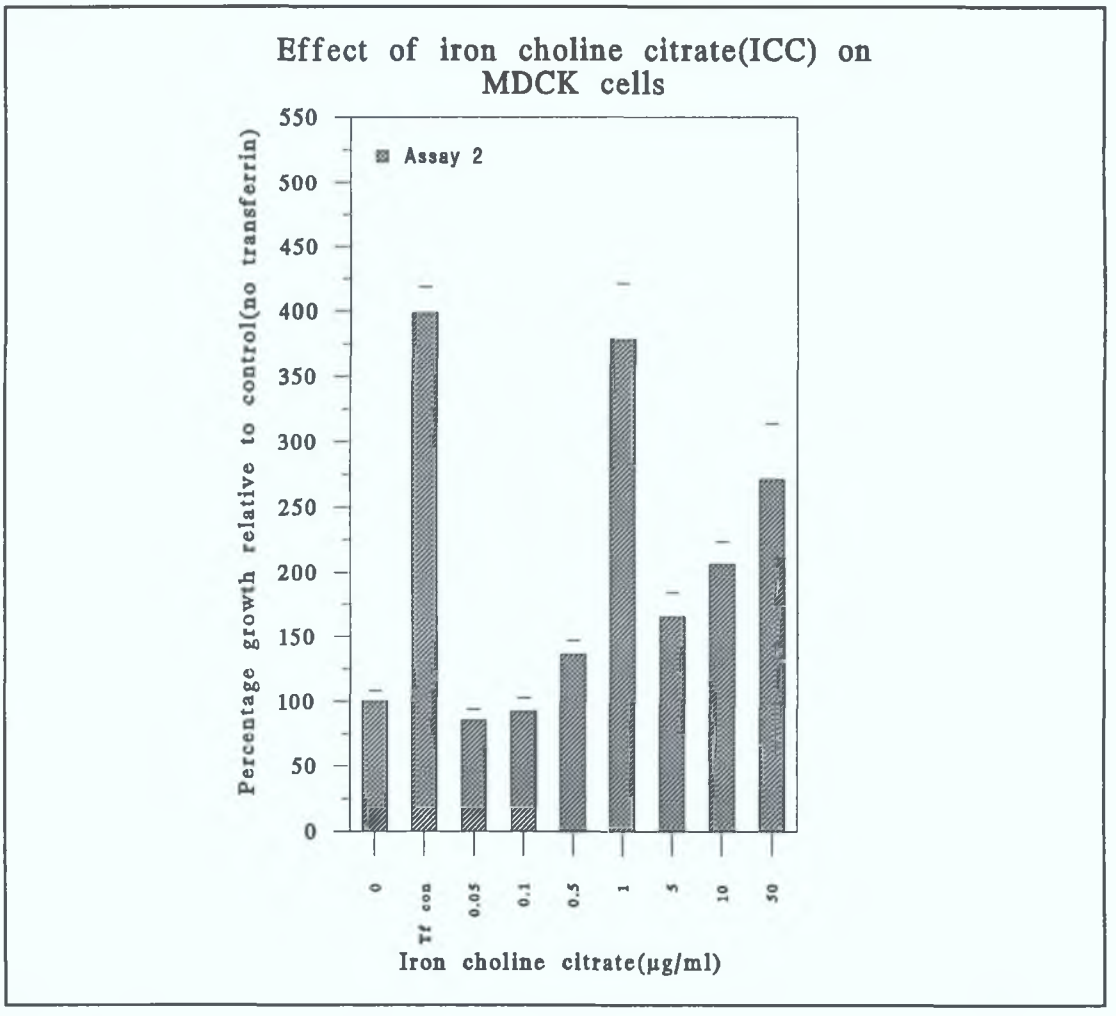


Figure 3.3.2.3 shows the growth response of MDCK cells to iron choline citrate (ICC) under serum-free conditions. The results are expressed as the average percentage growth relative to control (no transferrin) $\pm$ standard deviation (n=8). Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3.3.2.3. Tf con refers to the recommended concentration of 5µg/ml partially-saturated transferrin.

Table 3.3.2.3 Growth response to ICC

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0.0 µg/ml	100.0 $\pm$ 8.64	100.0 $\pm$ 8.30	100.0 $\pm$ 8.54
Tf con	422.9 $\pm$ 40.1	398.1 $\pm$ 20.5	344.8 $\pm$ 19.8
+ 0.05 µg/ml	98.09 $\pm$ 6.37	85.87 $\pm$ 7.81	82.76 $\pm$ 4.31
+ 0.1 µg/ml	101.3 $\pm$ 7.01	292.6 $\pm$ 10.0	86.20 $\pm$ 4.31
+ 0.5 µg/ml	410.8 $\pm$ 31.2	136.1 $\pm$ 11.1	149.1 $\pm$ 21.5
+ 1.0 µg/ml	411.5 $\pm$ 34.4	378.2 $\pm$ 43.0	286.5 $\pm$ 21.9
+ 5.0 µg/ml	250.3 $\pm$ 29.9	165.2 $\pm$ 19.4	148.5 $\pm$ 12.9
+ 10.0 µg/ml	226.1 $\pm$ 25.5	206.8 $\pm$ 16.3	168.2 $\pm$ 16.3
+ 50.0 µg/ml	390.1 $\pm$ 13.4	271.5 $\pm$ 42.3	230.2 $\pm$ 22.4

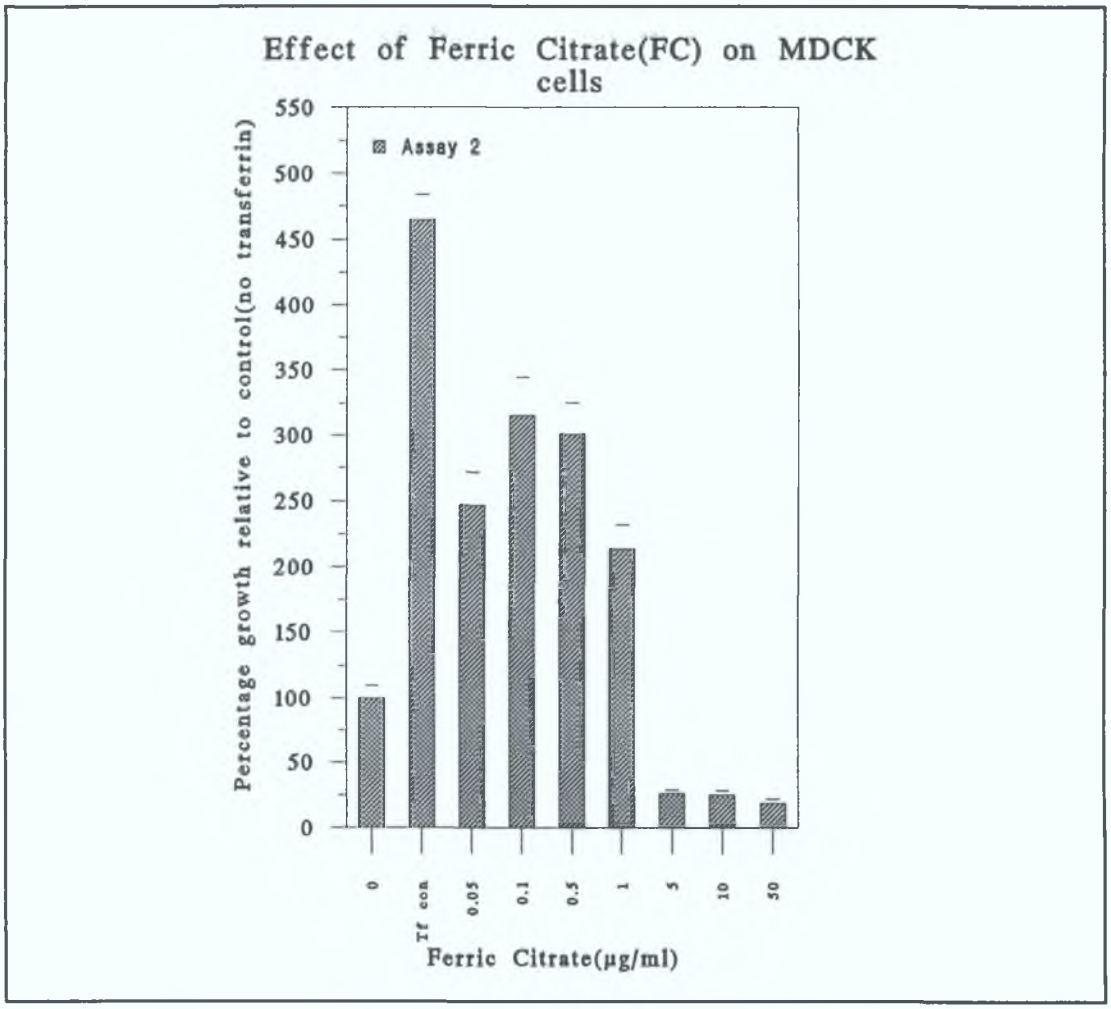


Figure 3.3.2.4 shows the growth response of MDCK cells to ferric citrate (FC) under serum-free conditions. The results are expressed as the average percentage growth relative to control (no transferrin) $\pm$ standard deviation (n=8). Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3.3.2.4. Tf con refers to the recommended concentration of 5μg/ml partially-saturated transferrin.

Table 3.3.2.4 Growth response to FC

Variables	ASSAY 1	ASSAY 2	ASSAY 3
0.0 μg/ml	100.0 $\pm$ 6.05	100.0 $\pm$ 9.50	100.0 $\pm$ 7.98
Tfcon	387.0 $\pm$ 35.8	464.9 $\pm$ 19.2	326.1 $\pm$ 33.5
+ 0.05 μg/ml	222.3 $\pm$ 15.8	246.9 $\pm$ 25.0	206.3 $\pm$ 20.5
+ 0.1 μg/ml	241.4 $\pm$ 26.5	315.6 $\pm$ 29.2	264.6 $\pm$ 25.2
+ 0.5 μg/ml	273.4 $\pm$ 27.8	301.3 $\pm$ 24.1	285.8 $\pm$ 20.5
+ 1.0 μg/ml	272.4 $\pm$ 9.17	214.1 $\pm$ 18.3	178.7 $\pm$ 16.5
+ 5.0 μg/ml	43.82 $\pm$ 5.50	25.87 $\pm$ 2.75	25.19 $\pm$ 2.36
+ 10.0 μg/ml	46.29 $\pm$ 6.80	25.00 $\pm$ 3.15	24.41 $\pm$ 2.36
+ 50.0 μg/ml	46.91 $\pm$ 4.30	19.05 $\pm$ 2.67	19.68 $\pm$ 1.57

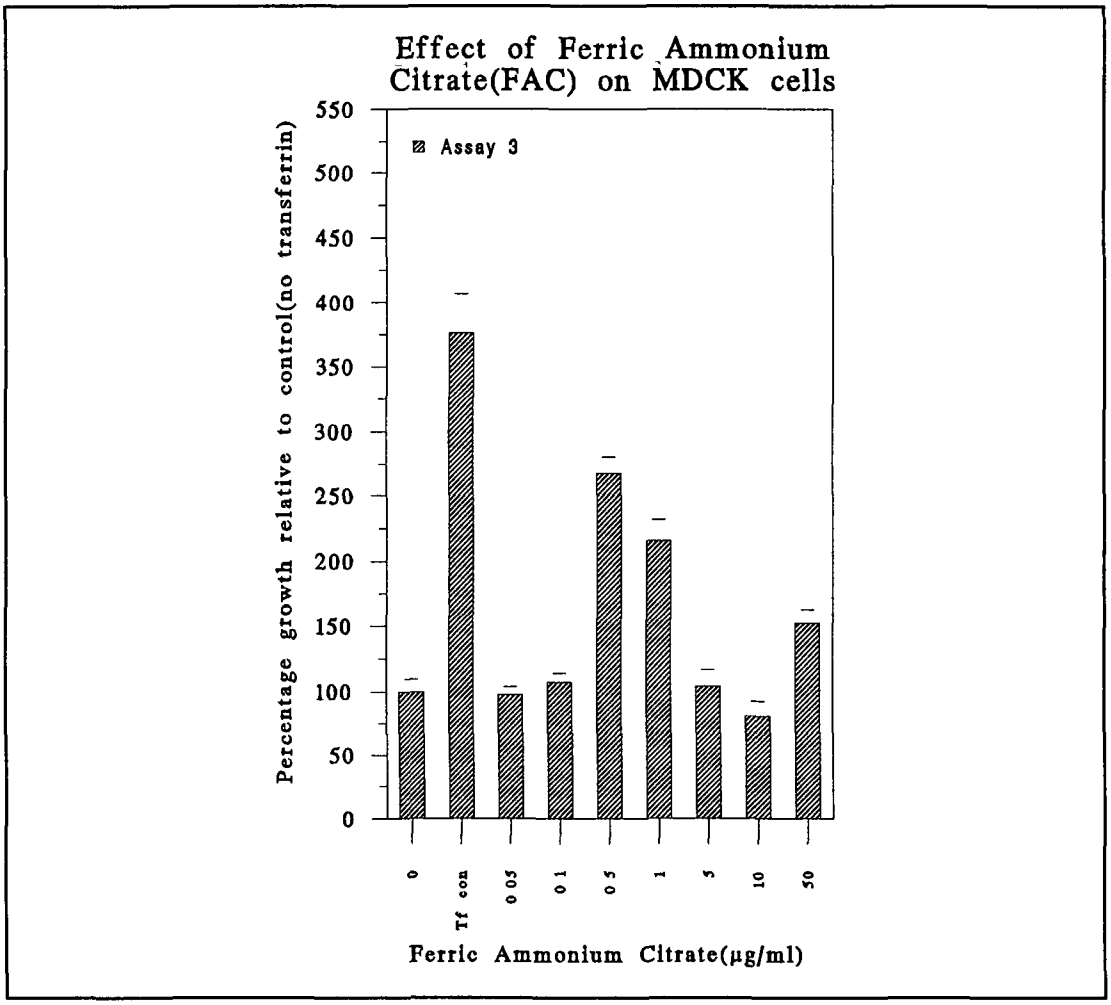


Figure 3.3.2.5 shows the growth response of MDCK cells to ferric ammonium citrate (FAC) under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3 2 5 Tf con refers to the recommended concentration of 5µg/ml partially-saturated transferrin

Table 3.3.2.5 Growth response to FAC

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 ± 13 2	100 0 ± 2 86	100 0 ± 9 11
Tf con	449 3 ± 49 6	435 2 ± 21 8	376 1 ± 30 8
+ 0 05 µg/ml	94 97 ± 8 19	102 7 ± 5 90	97 51 ± 5 97
+ 0 1 µg/ml	106 4 ± 5 73	112 2 ± 10 6	107 2 ± 6 91
+ 0 5 µg/ml	402 0 ± 31 9	283 3 ± 30 2	267 7 ± 12 9
+ 1 0 µg/ml	307 0 ± 27 2	232 7 ± 32 8	215 6 ± 16 4
+ 5 0 µg/ml	170 8 ± 20 3	97 05 ± 20 9	104 0 ± 12 9
+ 10 0 µg/ml	207 9 ± 17 2	130 7 ± 14 8	80 93 ± 11 5
+ 50 0 µg/ml	280 8 ± 22 1	125 4 ± 12 5	152 7 ± 10 2

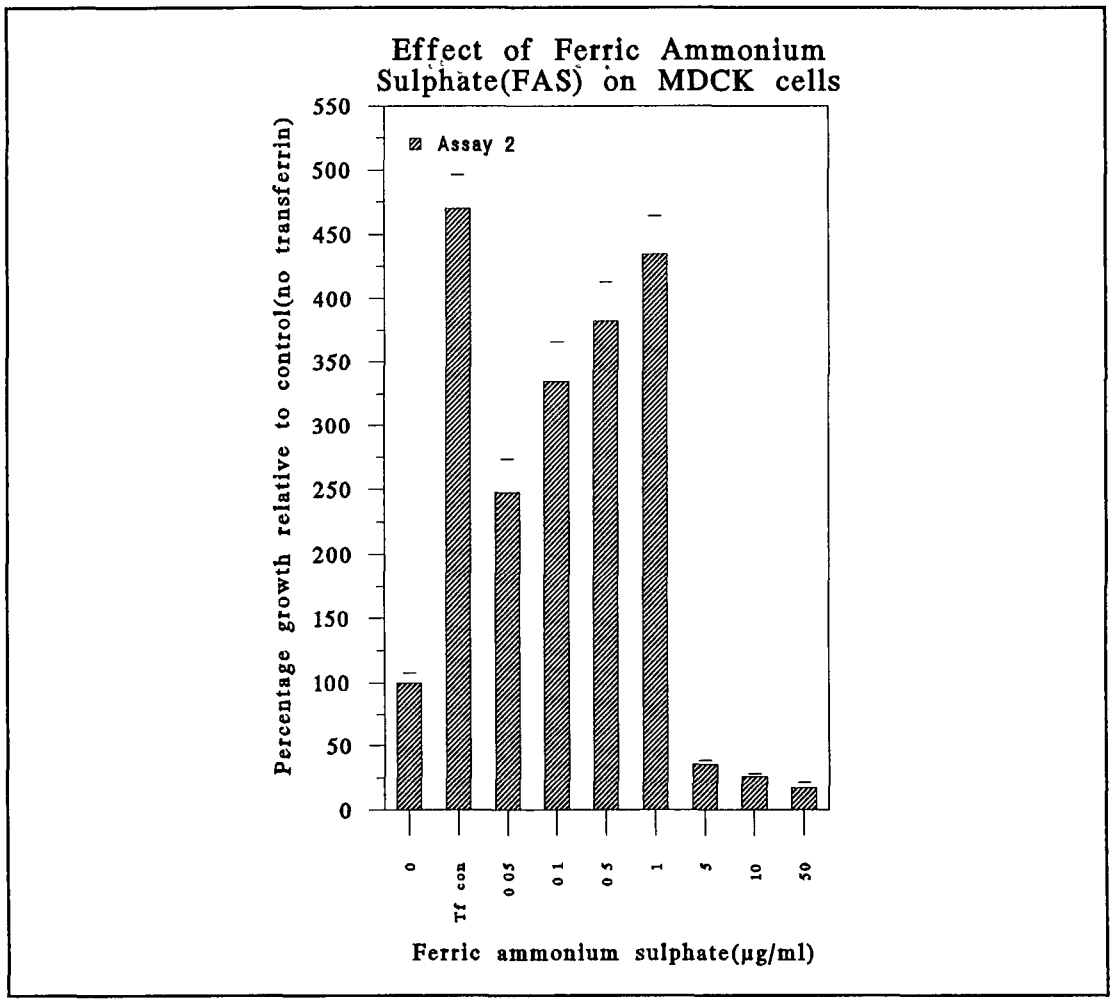


Figure 3.3.2.6 shows the growth response of MDCK cells to ferric ammonium sulphate (FAS) under serum-free conditions The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 3.2 6 Tf con refers to the recommended concentration of 5µg/ml partially-saturated transferrin

Table 3.3.2.6 Growth response to FAS

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 µg/ml	100 0 ± 6 35	100 0 ± 7 58	100 0 ± 7 77
Tf con	461 0 ± 44 6	470 0 ± 26 2	351 0 ± 23 4
+ 0 05 µg/ml	155 9 ± 14 2	247 6 ± 25 5	187 6 ± 17 1
+ 0 1 µg/ml	278 1 ± 17 5	334 5 ± 30 9	224 2 ± 14 8
+ 0 5 µg/ml	403 2 ± 37 4	382 1 ± 30 9	298 2 ± 24 1
+ 1 0 µg/ml	444 4 ± 33 3	434 5 ± 29 8	302 5 ± 6 91
+ 5 0 µg/ml	85 53 ± 8 18	35 42 ± 3 10	48 60 ± 4 20
+ 10 0 µg/ml	37 73 ± 2 58	25 99 ± 1 90	21 67 ± 3 09
+ 50 0 µg/ml	24 53 ± 3 14	17 38 ± 3 82	13 00 ± 1 78

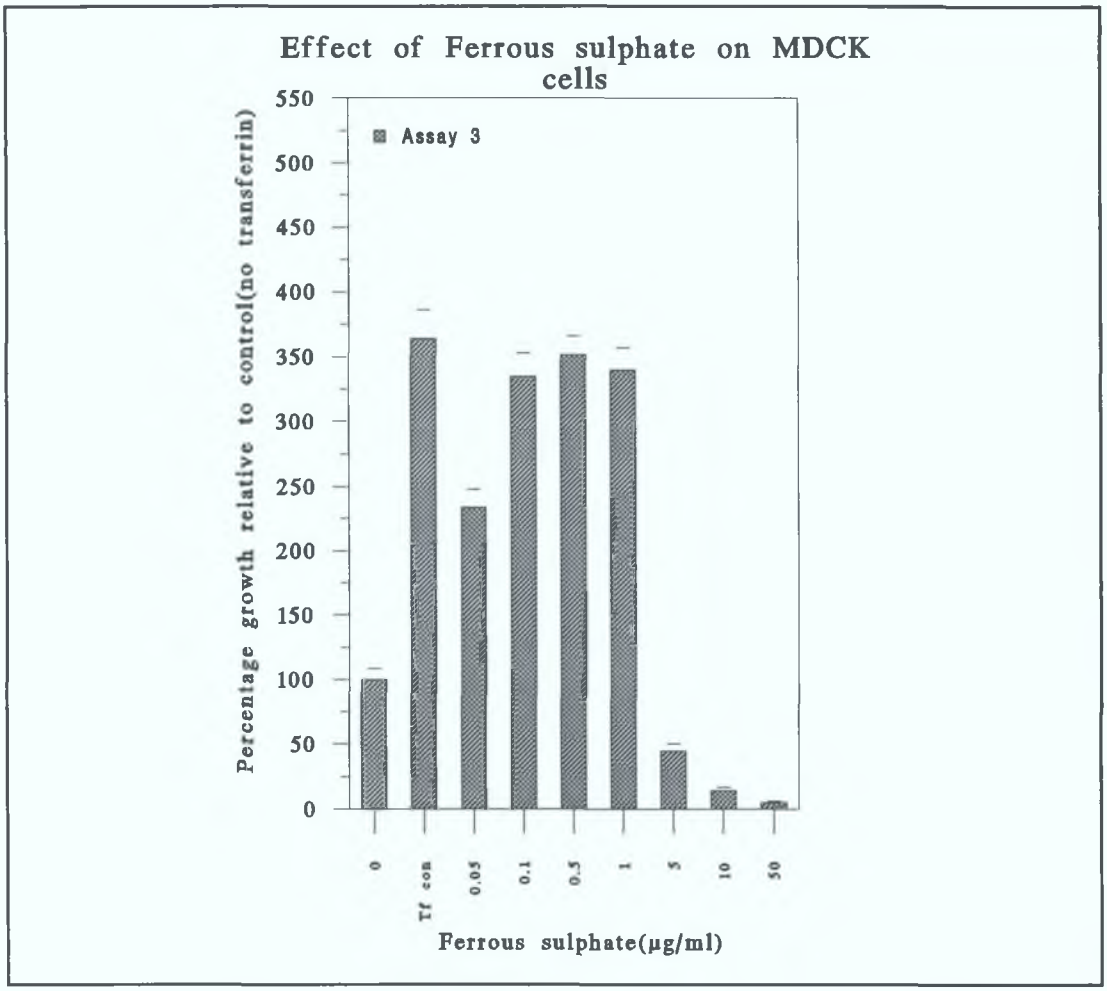


Figure 3.3.2.7 shows the growth response of MDCK cells to ferrous sulphate (Fe_2SO_4) under serum-free conditions. The results are expressed as the average percentage growth relative to control (no transferrin) $\pm$ standard deviation (n=8). Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3.3.2.7. Tf con refers to the recommended concentration of 5μg/ml partially-saturated transferrin.

Table 3.3.2.7 Growth response to Fe_2SO_4

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0.0 μg/ml	100.0 $\pm$ 6.15	100.0 $\pm$ 7.76	100.0 $\pm$ 8.36
Tf con	377.5 $\pm$ 32.4	435.2 $\pm$ 33.8	364.0 $\pm$ 22.4
+ 0.05 μg/ml	210.8 $\pm$ 8.92	269.2 $\pm$ 19.9	233.9 $\pm$ 14.0
+ 0.1 μg/ml	255.4 $\pm$ 23.9	342.7 $\pm$ 31.1	334.4 $\pm$ 18.3
+ 0.5 μg/ml	484.9 $\pm$ 22.1	371.8 $\pm$ 30.2	351.3 $\pm$ 14.7
+ 1.0 μg/ml	461.8 $\pm$ 34.3	321.9 $\pm$ 15.4	340.0 $\pm$ 16.9
+ 5.0 μg/ml	99.84 $\pm$ 9.43	41.66 $\pm$ 4.52	44.66 $\pm$ 5.32
+ 10.0 μg/ml	37.02 $\pm$ 2.43	20.83 $\pm$ 2.08	14.05 $\pm$ 2.89
+ 50.0 μg/ml	13.55 $\pm$ 1.37	4.61 $\pm$ 2.16	4.92 $\pm$ 0.89

3.3.3 SUBCULTURE OF MDCK CELLS WITH TRANSFERRIN REPLACEMENTS

In order to see if any of these iron complexes were able to replace transferrin it was necessary to see if the growth stimulatory effect was continued over a period of time By passaging cells, residual transferrin present in the cells would be lost revealing the true ability of the iron compounds to replace transferrin Of the iron containing compounds and salts, the data of section 3 3 2 indicated that the most promising were Fe_2SO_4, FAS, ICC and SNP since these gave stimulation in growth assays which was almost equal to that seen with the bovine transferrin control

MDCK cells were passaged 9 times in serum-free medium with transferrin or a replacement The results are shown in Figure 3 3 3 1 In the first subculture experiment, ICC and Fe_2SO_4 were found not to be suitable replacements The stimulatory ability became more reduced the longer the cells were subcultured, until no growth occurred in the eighth subculture and insufficient cells were available to reset up fresh flasks Microscopic observations showed the cells to be more extended than the cells in transferrin The cells looked unhealthy, being very granular For the two other replacements, SNP and FAS, good growth was sustained over 9 passages The growth was comparable to that obtained by transferrin

The inability of ICC and Fe_2SO_4 to support growth in a long-term culture may have been due to the loss of residual transferrin which was present in earlier passages as a serum contaminant within the cells and mediated transport of ICC and Fe_2SO_4 into the cells It was also possible that the age of the iron solutions may have allowed oxidation reactions to occur and result in a form of iron unsuitable for uptake by the cells In order to find out why these factors were effective in growth assays but not in long-term growth in transferrin-free medium, fresh solutions of the most interesting iron complexes were made up and used to passage the cells The results are shown in Table 3 3 3 2

The results showed that ICC was again unable to support growth over an extended time in transferrin-free medium Unfortunately, this subculture became contaminated in the fourth passage The inability to support growth would indicate that the effects seen in the growth assay in section 3 3 2 3 were due to residual tranferrin (in the cells) facilitating the uptake of iron Fe_2SO_4 was almost as effective as transferrin in this experiment indicating that the oxidation state of Fe_2SO_4 may affect its ability to replace transferrin FAS appeared to be the best replacement for transferrin from this subculture SNP was not as stimulatory

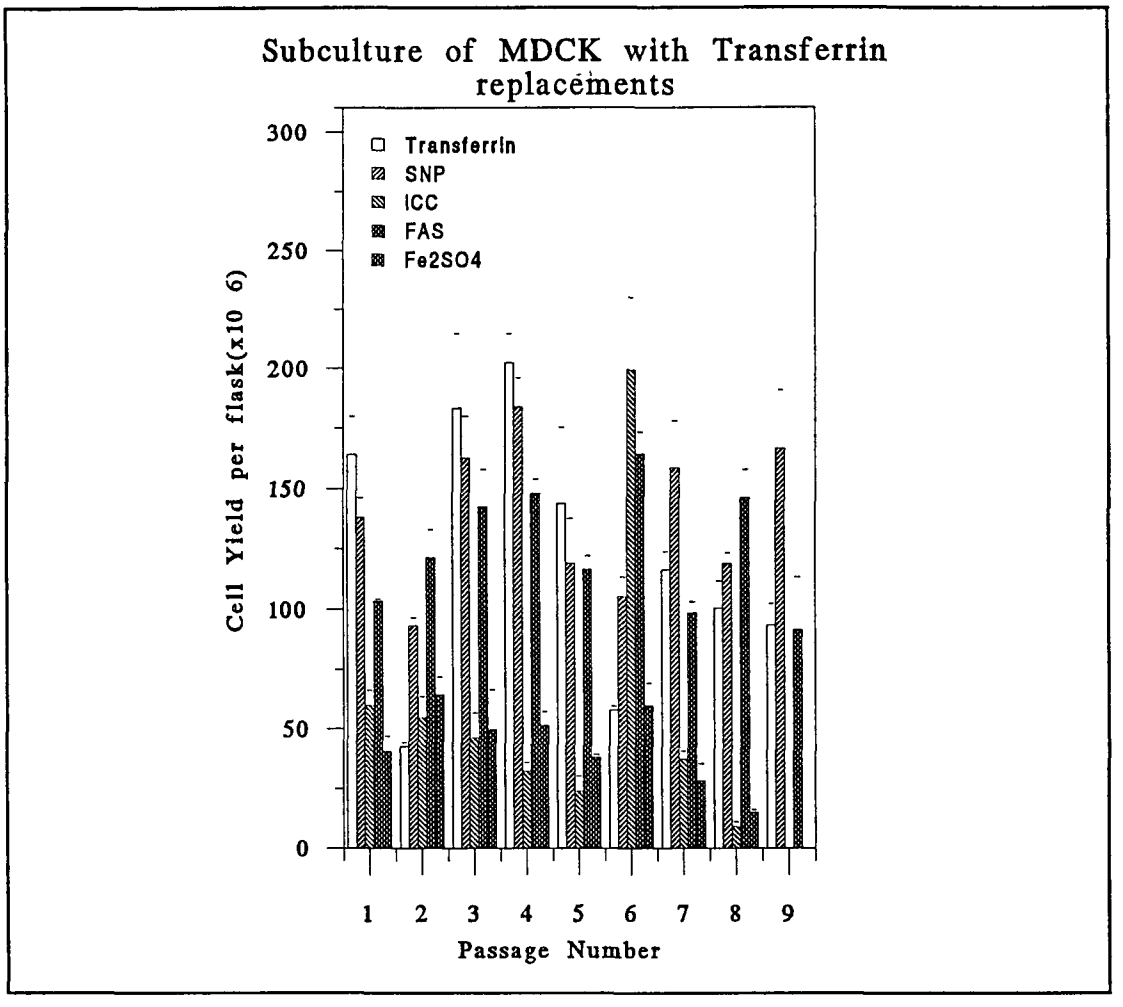


Figure 3.3.3 shows the growth response of MDCK cells to transferrin and transferrin replacements in serum-free medium The results are expressed as the cell yield per 25cm^2 flask (x10^{-4}) $\pm$ standard deviation (n=3) Cell yield was determined by haemocytometer counts and results are shown in Table 3 3 3

Table 3.3.3 Subculture of MDCK cells in serum-free medium with transferrin or alternatives

PASSAGE	TF	SNP	ICC	FAS	FE_2SO_4
1	164 1 $\pm$ 15 7	138 0 $\pm$ 8 04	59 60 $\pm$ 6 5	103 7 $\pm$ 0 77	40 66 $\pm$ 6 42
2	42 50 $\pm$ 1 86	93 25 $\pm$ 3 20	54 58 $\pm$ 9 08	121 6 $\pm$ 11 4	64 25 $\pm$ 7 52
3	183 1 $\pm$ 31 6	162 7 $\pm$ 17 2	46 26 $\pm$ 10 4	142 5 $\pm$ 15 6	49 87 $\pm$ 16 5
4	202 2 $\pm$ 12 6	183 9 $\pm$ 12 1	32 25 $\pm$ 3 77	148 0 $\pm$ 5 86	51 50 $\pm$ 5 70
5	143 7 $\pm$ 31 5	119 2 $\pm$ 18 4	24 00 $\pm$ 6 24	116 8 $\pm$ 5 39	38 25 $\pm$ 1 06
6	57 75 $\pm$ 1 71	105 2 $\pm$ 8 14	199 3 $\pm$ 30 8	164 4 $\pm$ 8 94	59 67 $\pm$ 9 36
7	116 5 $\pm$ 7 45	158 6 $\pm$ 19 5	37 25 $\pm$ 3 52	98 50 $\pm$ 4 64	28 25 $\pm$ 7 24
8	100 4 $\pm$ 11 2	118 9 $\pm$ 4 35	9 05 $\pm$ 1 97	146 0 $\pm$ 11 7	15 12 $\pm$ 1 11
9	93 19 $\pm$ 9 05	116 4 $\pm$ 24 2	———	91 35 $\pm$ 22 0	———

Table 3.3.3.2 Subculture of MDCK cells in SFM with transferrin (TF) replacements

PASSAGE	Tf	SNP	ICC	FAS	Fe_2SO_4
1	264 3 ± 1 94	183 5 ± 9 20	14 20 ± 4 20	216 7 ± 11 2	175 3 ± 10 4
2	144 0 ± 21 8	153 5 ± 45 9	5 20 ± 1 52	170 4 ± 8 50	156 6 ± 34 6
3	177 5 ± 17 7	130 0 ± 7 80	-----	200 0 ± 36 9	161 2 ± 7 10

Results are expressed as the Cell yield x 10^{-4} cells/25cm^2 flask ± standard deviation (n=3)

Due to the conflicting results obtained between the first and second subculture experiments, it was necessary to repeat the experiment The results for the final subculture are shown in Table 3 3 3 3 The growth in the first passage in SFM with the transferrin replacements was very low This was most likely due to the fact that when set up in the first passage, cells were in the late stage of exponential growth and would have had a longer lag phase than cells which were in mid-exponential phase Again ICC was found to be unable to support growth through more than 3 passages Fe_2SO_4 was able to support growth up to passage 5 when there appeared to be a slight loss in stimulation in comparison to transferrin and the other factors

Cells from Fe_2SO_4-containing SFM at the end of passage 5 were split as in earlier passages, but one set of flasks were exposed to the same stock of Fe_2SO_4 (referred to as Fe_2SO_4 old) while the second set were exposed to freshly made up Fe_2SO_4 (referred to as Fe_2SO_4 new) It was not until the last passage, that a difference in the response of the two solutions was seen, where freshly made up Fe_2SO_4 was more stimulatory to the cells This may indicate the possibility that Fe_2SO_4 in the first subculture was in an oxidized form which was not available to the cells

Table 3.3.3.3 Subculture of MDCK cells in SFM with transferrin (TF) replacements

PASSAGE	TF	SNP	ICC	FAS	Fe_2SO_4
1	43 0 ± 9 12	32 80 ± 4 50	25 00 ± 6 73	18 70 ± 0 35	20 70 ± 0 25
2	142 0 ± 17 6	120 0 ± 15 6	51 60 ± 6 10	110 0 ± 14 0	53 00 ± 4 00
3	85 00 ± 36 0	76 80 ± 14 3	17 60 ± 5 57	79 20 ± 15 8	133 1 ± 13 2
4	206 7 ± 89 0	116 0 ± 5 66	-------	133 8 ± 4 32	109 0 ± 11 3
5	76 90 ± 9 00	189 4 ± 22 8	Fe_2SO_4 New	60 80 ± 6 50	46 20 ± 3 50
6	50 00 ± 7 00	79 50 ± 13 4	136 7 ± 16 6	112 3 ± 8 90	114 7 ± 29 0
7	172 1 ± 11 4	73 10 ± 15 4	69 50 ± 2 30	86 75 ± 3 90	75 60 ± 20 0
8	106 5 ± 5 30	130 5 ± 7 80	151 0 ± 1 59	148 9 ± 15 9	192 7*
9	199 9 ± 28 1	199 5 ± 39 0	183 7 ± 4 24	196 9 ± 18 6	123 4 ± 15 2

Results are expressed as the Cell yield x 10^{-4} cells/25cm^2 flask ± standard deviation (n=3)

In summary, it was possible to replace transferrin with SNP or FAS in SFM Fe_2SO_4 was also a possible replacement for transferrin but it would be necessary to make up fresh stocks every 3 - 4 weeks ICC was not suitable a suitable replacement for transferrin over a long-term period

With the low stimulatory effect of insulin (Appendix D) and the ability to replace natural PGE with synthetic PGE or di-butyryl-cAMP (Taub *et al* , 1984), it is possible to grow MDCK cells in a SFM devoid of animal-derived products

3.4 EFFECT OF INSULIN ON GROWTH OF CHOK1 CELLS IN SFM

Insulin is, in general, one of the major components of serum-free media The use of insulin at microgram/ml levels which are supraphysiological (normal blood levels are 4 to 30μU/ml), has led to investigations into the biological activity of insulin For many cell types, insulin was found to exert a mitogenic effect only at high concentrations because it was cross-reacting with the IGF-I receptor For a small number of cell lines, the insulin was found to be active at nanogram levels, *via* its own receptor

In growing CHOK1 and MDCK cells in serum-free media, it was found that CHOK1 cells were very responsive to insulin Mendiaz *et al* (1986) found that CHOK1 cells showed a significant increase in growth rate at insulin concentrations of 1 to 5ng/ml There after, the growth rate was more gradual and reached a saturation point at 10μg/ml In support of this, Mamounas *et al* (1989) found that insulin acted at its own receptor with a half maximum stimulation of 14ng/ml In addition, it was found that IGF-I had little or no effect on DNA synthesis until concentrations of 225ng/ml, while IGF-II showed some stimulation of DNA synthesis with a half maximum activity at 75ng/ml

Mendiaz also presented information on the existence of msulin-independent mutants, which could become predominant in the serum-free medium cited without the presence of insulin However, the morphological descriptions of the cells under serum-free with and without insulin were at odds with the observations made here Mendiaz observed that CHOK1 cells grown in the serum- free medium cited, without insulin were elongated and fibroblastic in nature, while in the presence of insulin, the cells appeared round to cuboid
The CHOK1 cells in this laboratory were found to be fibroblastic in shape both with and without insulin present Only when 80 - 90% confluency was reached, did cells round up or when cells were inoculated at an exceedingly low density At a low seeding density, cells rounded up, formed clusters and detached from the surface of the plate

The aim of this section was to investigate (as Mendiaz did) whether insulin could be replaced by nanogram levels of either IGF-I or IGF-II In addition, a comparison of bovine-derived insulin and insulins from other sources were compared with the intention of replacing the bovine-derived msulin with a recombinant insulin As microscopic observations of the cells in serum-free medium with and without insulin were apparently different to the CHOK1 cells

grown by Mendiaz, it was of interest to see if the response of the CHOK1 cells used in these studies, to IGF-I and IGF-II was similar to that obtained by Mendiaz

If, either of the IGFs could replace insulin, then the serum-free medium designed initially be Mendiaz might (if the insulin concentration were appropriate) contain a lower protein concentration As transferrin was not essential for the cells (as shown here and by Mendiaz), the only exogenous protein being added to the Ham's F12 would be the insulm/IGFs The use of recombinant IGFs or insulin would mean that no animal-derived factors would be needed for growth of CHOK1 cells in SFM

Cells were grown for one subculture (4 days) in serum-free medium as described in section 3 2 1 Assays were set up in 96-well plates and acid phosphatase was used as the end point All assays were set up with 2 controls on each plate The negative control contained all the factors in the medium designed by Mendiaz (1986) except insulin The positive control which is referred to as the Ins Con in all the Figures, contains all the factors in the medium designed by Mendiaz (1986) with bovine insulin at 10μg/ml

3.4.1 COMPARISON OF INSULIN AND IGFs ON CHOK1 CELLS IN SFM

In this part, bovine insulin was compared to the IGFs on a molar basis, to see how well mole for mole each stimulated the growth of CHOK1 cells in serum-free medium As mentioned earlier, insulin is one of the most commonly used factors in SFM Its growth stimulatory activity was found in many cases to be due to the insulin (at supraphysiological concentrations) binding and activating the IGF-I receptor For this reason, a molar basis of comparison was chosen Table 3 4 1 shows the relationship between molarity and nanogram levels for bovine insulin and the IGFs

Table 3.4 1

COMPONENT	MOLECULAR WEIGHT	EQUIVALENT CONCENTRATION TO 10nM
Insulin	5,733 5	57 3ng/ml
IGF-I	7,600	76ng/ml
IGF-II	7,500	75ng/ml

The results are shown in Figures 3 4 1 1 to 3 4 1 3 The results for bovme insulin are shown in Figure 3 4 1 1 A maximum stimulation was observed at 10nM (57 3ng/ml) The stimulation at this concentration was comparable to that of the serum-free control which contains 10μg/ml bovme insulin Thereafter, the insulin begins to loose its stimulatory ability The

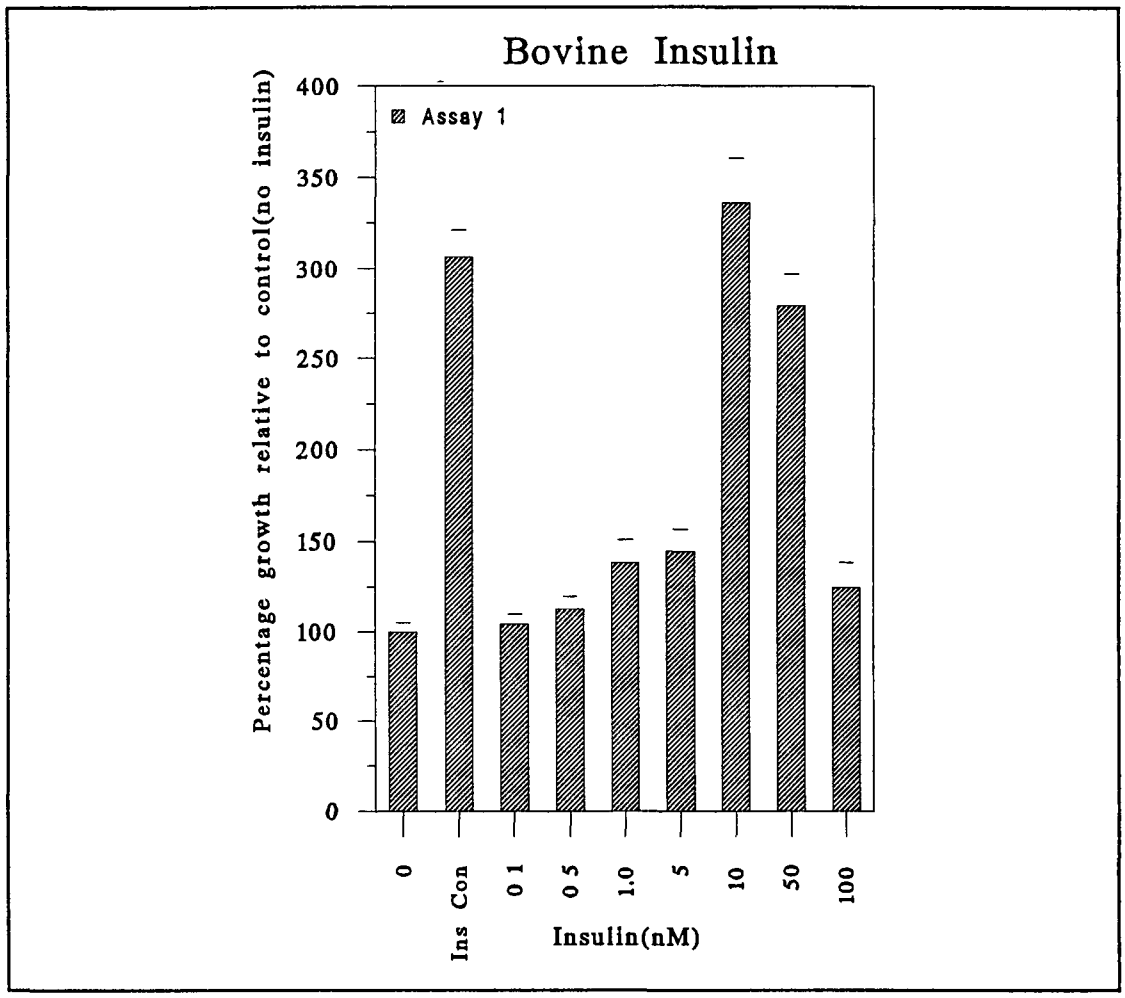


Figure 3.4.1.1 shows the growth response of CHOK1 cells to bovine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 4 1 1

Table 3 4 1 1 Growth response to bovine insulin

VARIABLES		ASSAY 1	ASSAY 2
0 0nM Insulin	0 0ng/ml Insulin	100 0 ± 8 92	100 0 ± 5 0
Ins Con	Ins Con	331 4 ± 31 8	306 8 ± 14 2
+ 0 1nM	+ 0 57ng/ml	101 1 ± 8 89	104 0 ± 5 50
+ 0 5nM	+ 2 7ng/ml	114 7 ± 9 83	112 5 ± 7 00
+ 1 0nM	+ 5 73ng/ml	119 3 ± 8 23	138 2 ± 12 8
+ 5 0nM	+ 27ng/ml	227 9 ± 12 1	144 3 ± 12 0
+ 10 0nM	+ 57 3ng/ml	343 1 ± 20 6	335 9 ± 24 8
+ 20 0nM	+ 0 1147μg/ml	299 8 ± 24 0	279 1 ± 17 7
+ 50 0nM	+ 0 27μg/ml	181 7 ± 26 5	124 5 ± 13 9
+ 100nM	+ 0 573μg/ml	96 88 ± 4 21	51 86 ± 4 15

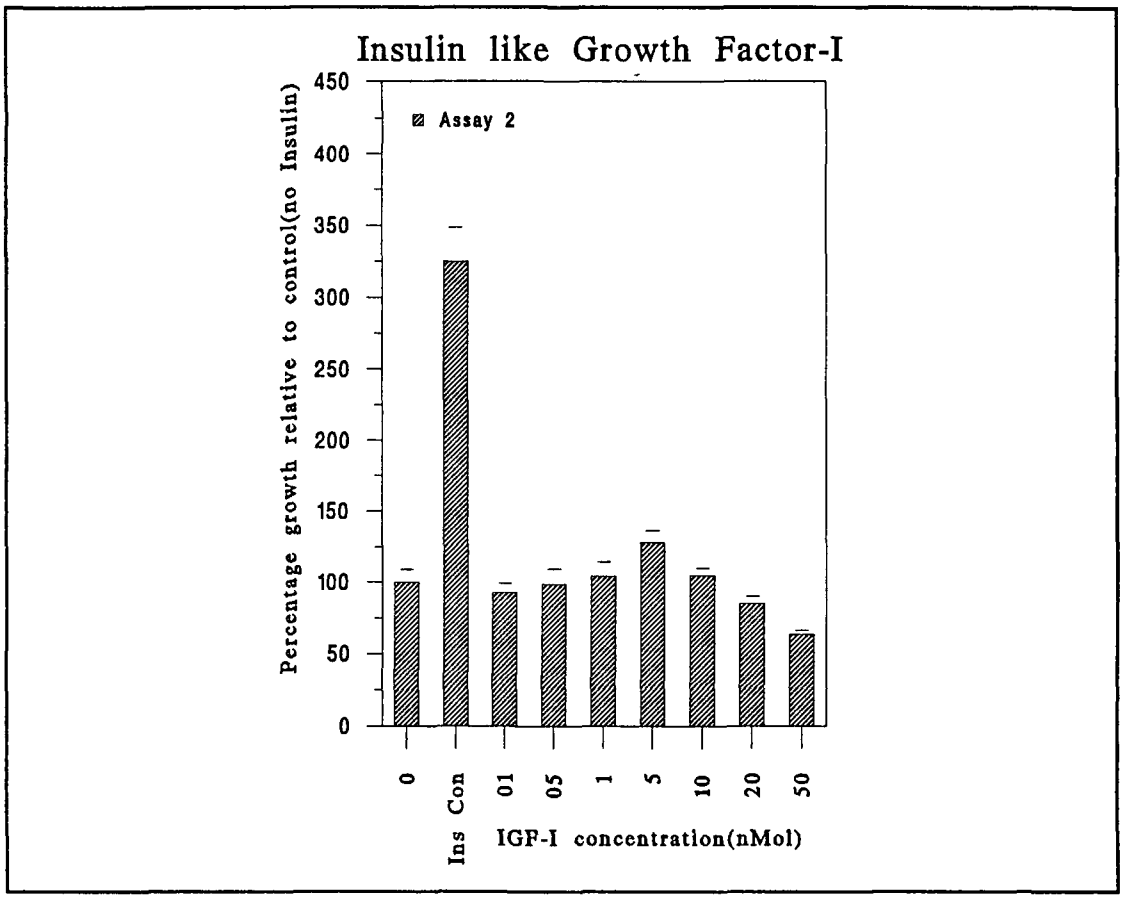


Figure 3.4.1.2 shows the growth response of CHOK1 cells to IGF-I under serum-free conditions. The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 1 2

Table 3 4 1 2 Growth response to IGF-I

IGF-I		ASSAY 1	ASSAY 2	ASSAY 3
nM	ng/ml			
0 0 nM	0 0 ng/ml	100 0 ± 7 33	100 0 ± 8 52	100 0 ± 5 41
Ins Con	Ins Con	270 9 ± 25 3	325 0 ± 23 6	310 3 ± 26 8
+ 0 1 nM	+ 0 76 ng/ml	99 54 ± 4 61	92 81 ± 6 30	96 87 ± 8 21
+ 0 5 nM	+ 3 8 ng/ml	105 5 ± 5 99	98 84 ± 10 3	97 87 ± 5 33
+ 1 0 nM	+ 7 6 ng/ml	139 4 ± 9 55	104 2 ± 10 3	111 2 ± 12 5
+ 5 0 nM	+ 38 ng/ml	144 1 ± 9 28	128 0 ± 8 10	94 83 ± 6 51
+ 10 0 nM	+ 76 ng/ml	108 6 ± 17 9	104 8 ± 4 86	68 33 ± 3 29
+ 20 0 nM	+ 152 ng/ml	98 52 ± 9 80	85 08 ± 4 86	61 29 ± 2 19
+ 50 0 nM	+ 380 ng/ml	57 14 ± 6 54	63 73 ± 2 70	55 82 ± 4 92
+ 0 0 nM	+ 0 0 ng/ml	103 2 ± 6 91	104 2 ± 4 32	101 8 ± 6 57

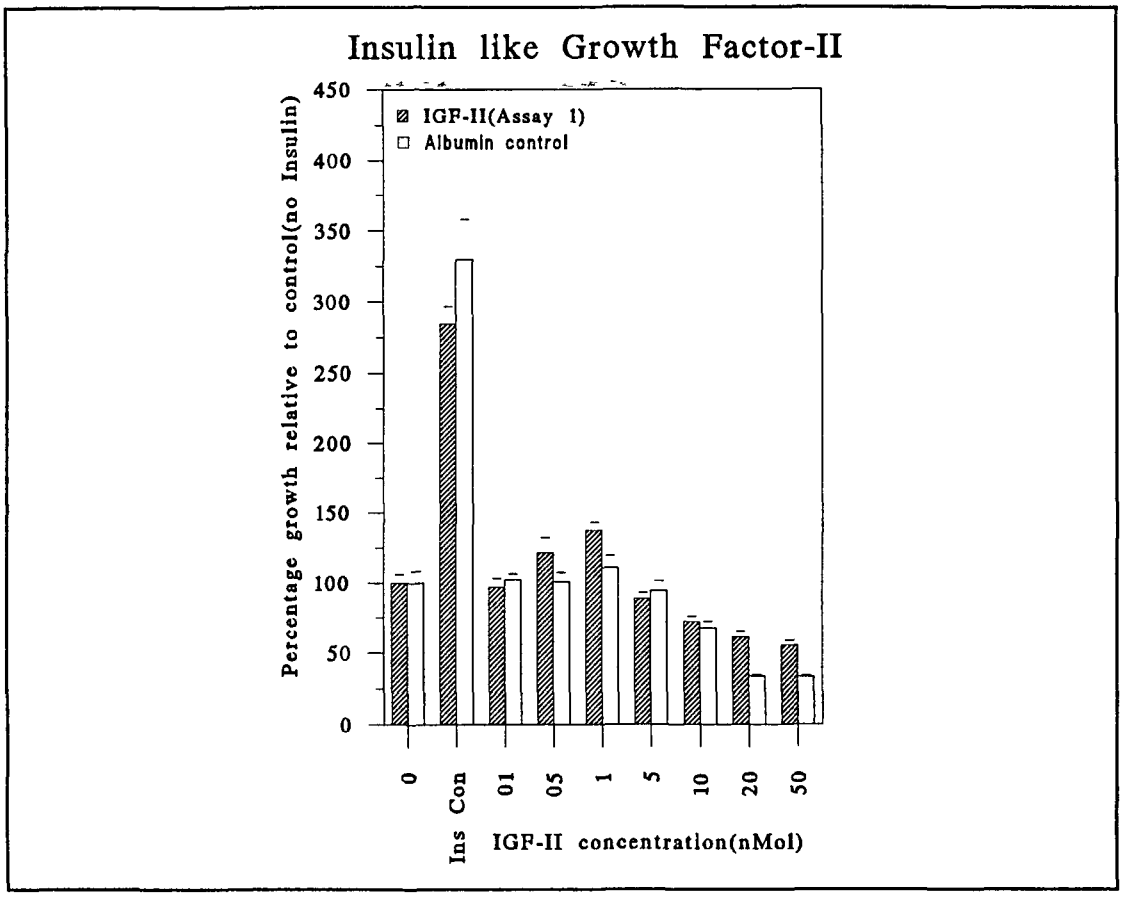


Figure 3.4.1.3 shows the growth response of CHOK1 cells to IGF-II under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Tables 3 4 1 3a to c.

Table 3 4 1 3a Growth response to IGF-II

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3
0 0nM	0 0ng/ml	100 0 ± 5 70	100 0 ± 8 24	100 0 ± 8 69
Ins Con	Ins Con	284 4 ± 12 2	282 9 ± 18 9	248 3 ± 12 6
+ 0 1nM	+ 0 75ng/ml	96 79 ± 6 36	94 49 ± 7 58	96 96 ± 7 23
+ 0 5nM	+ 3 75ng/ml	121 8 ± 10 5	92 58 ± 6 42	96 13 ± 9 96
+ 1 0nM	+ 7 5ng/ml	137 7 ± 5 41	94 88 ± 7 33	104 8 ± 3 28
+ 5 0nM	+ 37 5ng/ml	88 53 ± 4 20	120 1 ± 9 24	108 7 ± 6 28
+ 10 0nM	+ 75ng/ml	72 48 ± 3 67	113 1 ± 6 65	103 9 ± 7 25
+ 20 0nM	+ 150ng/ml	61 93 ± 3 39	67 38 ± 4 22	105 8 ± 5 79
+ 50 0nM	+ 375ng/ml	55 88 ± 3 29	56 38 ± 5 50	114 6 ± 12 0
+ 0 0nM	+ 0 0ng/ml	101 4 ± 6 42	100 4 ± 8 25	106 6 ± 6 92

Table 3 4 1 3b Growth response to Albumin control

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0nM	100 0 ± 7 92	100 0 ± 7 13	100 0 ± 6 47
Ins Con	329 5 ± 28 4	318 1 ± 11 3	324 7 ± 28 6
+ 0 1nM	102 2 ± 4 37	93 75 ± 3 98	100 7 ± 7 22
+ 0 5nM	100 8 ± 6 52	89 77 ± 4 54	98 24 ± 9 25
+ 1 0nM	110 4 ± 8 74	88 39 ± 8 02	101 1 ± 5 63
+ 5 0nM	93 99 ± 7 10	77 84 ± 5 68	85 02 ± 6 16
+ 10 0nM	67 68 ± 4 84	67 61 ± 4 54	70 92 ± 4 44
+ 20 0nM	33 88 ± 1 09	60 22 ± 0 51	60 79 ± 3 08
+ 50 0nM	33 33 ± 1 09	51 93 ± 1 61	50 77 ± 0 22
+ 0 0nM	104 4 ± 7 10	97 08 ± 2 16	106 2 ± 7 05

Table 3 4 1 3c IGF-II - Albumin control

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0nM IGF-II	100 0 ± 7 92	100 0 ± 7 13	100 0 ± 6 47
+ 0 1nM	94 70 ± 6 22	100 8 ± 8 08	98 69 ± 7 18
+ 0 5nM	120 8 ± 10 4	103 1 ± 7 15	95 08 ± 10 1
+ 1 0nM	124 7 ± 6 19	107 3 ± 8 29	123 3 ± 3 24
+ 5 0nM	94 52 ± 4 47	154 3 ± 11 9	153 3 ± 7 39
+ 10 0nM	107 1 ± 5 42	167 3 ± 9 84	170 9 ± 10 2
+ 20 0nM	182 8 ± 10 0	111 9 ± 7 01	174 0 ± 9 52
+ 50 0nM	167 7 ± 9 87	108 6 ± 10 6	225 7 ± 23 6

discrepancy between the lack of stimulation of insulin at microgram levels and the 10μg/ml control is discussed in section 4 3 (page 343)

The results showed that at the concentrations tested, IGF-I and IGF-II were not very stimulatory to cell growth (Figure 3 4 1 2 and 3 4 1 3) For IGF-I, variations in maximum stimulation occurred from experiment to experiment, but reached a maximum of 28 to 44% stimulation over the control (no insulin) at a concentration of 5nM (38ng/ml) in two of the three assays At higher concentrations (about 380ng/ml) the IGF became slightly inhibitory

In Figure 3 4 1 3, the effect of IGF-II appeared to be very low However, bovine serum albumin-fatty acid free was required to act as a stabilizer for the IGF-II The albumin alone was inhibitory to the cells and so any stimulatory effect of the IGF-II may have been masked Table 3 4 1 3c shows the stimulatory effect when the albumin was taken into account Stimulation was greater than that seen for the IGF-I Maximum stimulation varied from experiment to experiment reaching values of 167 3 to 225 7% stimulation relative to the control (no insulin) The concentration range in which the stimulation occurred between 5 and 50nM (37 5 and 375ng/ml) The half maximum activity for the three experiments were 109, 24 32 and 57 82ng/ml for experiments 1 to 3

These results correlate to the findings of Mamounas (1989), that insulin would appear not to exert its growth stimulatory effect through the IGF-I receptor If this had been so, IGF-I should have been as stimulatory at nanogram levels as insulin was at microgram levels

3.4.2 EFFECT OF THE INSULIN SOURCE ON CHOK1 CELLS IN SFM

In order to compare the growth of different sources of insulin with slight variations in molecular weight, it was decided to compare them on the basis of international units (IU) An international unit is defined as the activity contained in 0 04167mg of fourth international standard preparation of bioassay (Pharmacopia, 1958) The standard contains 52% bovine and 48% porcine insulin with an equivalent 24 units/mg In addition to bovine and recombinant human insulin, ovine, porcine and equine-derived insulins were also investigated Assays were set up using the procedure described in section 3 4 1 The relationships between international units and mg/ml concentrations are shown in Table 3 4 2 The results are shown in Figures 3 4 2 1 to 3 4 2 5

Table 3 4 2 International Units for each insulin

INSULIN SOURCE	INTERNATIONAL UNITS EQUIVALENCE
Bovine	25 7IU per mg
recombinant Human	28 3IU per mg
Ovine	23 2IU per mg
Porcine	26 1IU per mg
Equine	23 5IU per mg

3.4.2.1 Bovine Insulin

As seen in Figure 3 4 2 1, increasing the insulin concentration resulted in increased growth Maximum stimulation was seen in the range of 0 002 to 0 02IU/ml bovine insulin, with the highest stimulation at 0 01IU/ml This corresponded to 0 389μg/ml (25 7IU per mg protein) After 0 02IU/ml (0 77μg/ml), the extent of stimulation decreased with inhibition seen at 1IU/ml in 3 of the 5 assays The loss of stimulation or the inhibition seen in this range did not coincide with the stimulation seen with the control insulin (10μg/ml) As the insulin was initially dissolved in 1 5M HCl before dilution, a pH effect may have explained the loss in stimulation However, the pH of the highest concentration was pH 7 43 while that of the control was pH 7 35 This trend was seen with the other insulins

Microscopic analysis showed that high cell density corresponded to high acid phosphatase (AP) activity In the higher concentrations of msulm, where low AP activity was seen, little growth was observed little spreading and some clumping of cells was visible

Activity at the ng level was seen but the extent of activity in the 5 repeats was quite variable All assays were carried out using the same procedure and cells used in the assays were in the log phase of growth Gasser *et al* (1985) found the stimulation by msulm to be very variable However, the work was carried out by Gasser *et al* was with a CHO subclone and a different serum-free medium It may be that the ability of insulin to stimulate cell growth may depend on factors in the basal medium, which may change from batch to batch of medium Mendiaz, however, stated that the incorporation of trace elements in Ham's F12 was to ensure batch to batch consistency It may have been that some other component was being varied from batch to batch

For the 5 experiments, the extent of stimulation varied between 3-fold and 6-fold stimulation The half maximum activity of each run was 40, 17 5, 127, 47 and 103ng/ml for runs 1 to 5 respectively However, when the ratios of maximum stimulation were compared to the insulin control, the results appeared less variable For assays 1 to 5, ratios were 1 476, 1 588, 1 52, 1 02 and 1 21 respectively, with an average and standard deviation of 1 36 $\pm$ 0 238

Compared to the results obtained by Mendiaz, the insulin was less stimulatory overall and appeared to require a higher concentration to elicit the same effect Unlike Mendiaz, at the higher concentrations, some loss of stimulation or inhibition was observed in these experiments In addition, cells with or without insulin appeared elongated and fibroblastic like

3.4.2.2 Recombinant Human Insulin

Recombinant human insulin was growth stimulatory in a concentration-dependent manner The results are shown in Figure 3 4 2 2 Maximum stimulation was between 0 01 and 0 1IU/ml (concentration 0 283 to 2 83μg/ml) After this the recombinant insulin became less stimulatory Microscopic observations showed that strong AP production corresponded to good growth and spreading while low AP production corresponded to little growth (very little spreading and the presence of clumps of cells) Activity was seen at slightly higher the physiological concentrations of 0 01 to 0 02IU/ml (283 to 566ng/ml) In this range the recombinant human insulin was as good, if not better than the 10μg/ml bovine insulin control The half maximum activities for each experiment were very variable, being 52, 41, 127 5, 241 and 462ng/ml respectively Except for runs 4 and 5, the other half maximum activities occurred around the same concentration as those for the respective bovine insulin experiments
For assays 1 to 5 the ratios of maximum stimulation to the insulin control were 1 513, 1 377, 1 383, 0 899 and 0 981 (average and standard deviation of 1 231 $\pm$ 0 272)

3.4.2.3 Porcine Insulin

Porcine insulin stimulated growth in a concentration-dependent manner (Figure 3 4 2 3) at concentrations up to 0 002 to 0 02IU/ml A plateau effect was seen in 3 of 5 assays at 0 01IU/ml These concentrations correspond to 76 6 to 766ng/ml (26 1 IU per mg protein) Porcine insulin was slightly more effective at low physiological concentrations than bovine insulin This was reflected in the half maximum activity for each experiment (6 87, 26 55,

14 5, 54 8 and 68 85ng/ml) For assays 1 to 5, the ratio of maximum stimulation to the insulin control were 1 413, 1 237, 1 133, 0 951 and 1 176 (average and standard deviation of 1 168 $\pm$ 0 190) As with the bovine insulin, after 0 02IU/ml, the stimulatory activity fell sharply and became inhibitory (up to 60 - 70% inhibition at 1IU/ml) Again porcine insulin was initially dissolved in 1 5M HCl before dilution The pH of the highest concentration tested (1IU/ml) was pH 7 40

3.4.2.4 Equine Insulin

Equine insulin stimulated growth in a concentration-dependent manner as seen in Figure 3 4 2 4 There appeared to be a break through point at 0 01 to 0 02IU/ml (0 425 to 0 85μg/ml), at which concentration, the activity was lower or equal to that of the bovine msulm control The values for the half maximum activity for each run were 50 66, 126, 41 1, 333 and 593 5ng/ml For assays 1 to 5, the ratios of maximum stimulation to the insulin control were 1 002, 1 051, 0 520, 0 809 and 1 20 (average and standard deviation of 0 918 $\pm$ 0 262) Inhibition was seen at the higher concentrations In this range microscopic observations showed a decrease in the amount of cell spreading (and in the number of cells present) and an increase in the number of clumps of cells At 1IU/ml, the cells were rounded up and appeared as if they had just been inoculated onto the plate With a pH of 7 43, an acid medium could not explain the inhibition

3.4.2.5 Ovine Insulin

Ovine insulin stimulated growth in a concentration-dependent manner (Figure 3 4 2 5) with the best growth obtained between 0 002 and 0 02IU/ml (0 0862 and 0 862μg/ml) The growth in this range surpassed that achieved with the bovine msulm control at 10μg/ml At 0 1IU/ml (4 3μg/ml), the stimulatory effect was almost abolished and at higher concentrations became inhibitory

Microscopic observations showed the same trend as for equme insulin At the lower concentration range of 0 001 to 0 002IU/ml (0 0431 to 0 0862μg/ml) growth was better than that observed with the bovine msulm at the same concentration The values for the half maximum activity were 34 99, 22 46, 70 54, 529 and 222 9ng/ml For assays 1 to 5, the ratios of maximum stimulation to the insulin control were 1 41, 1 669, 1 307, 1 147 and 1 121 (average and standard deviation of 1 33 $\pm$ 0 223)

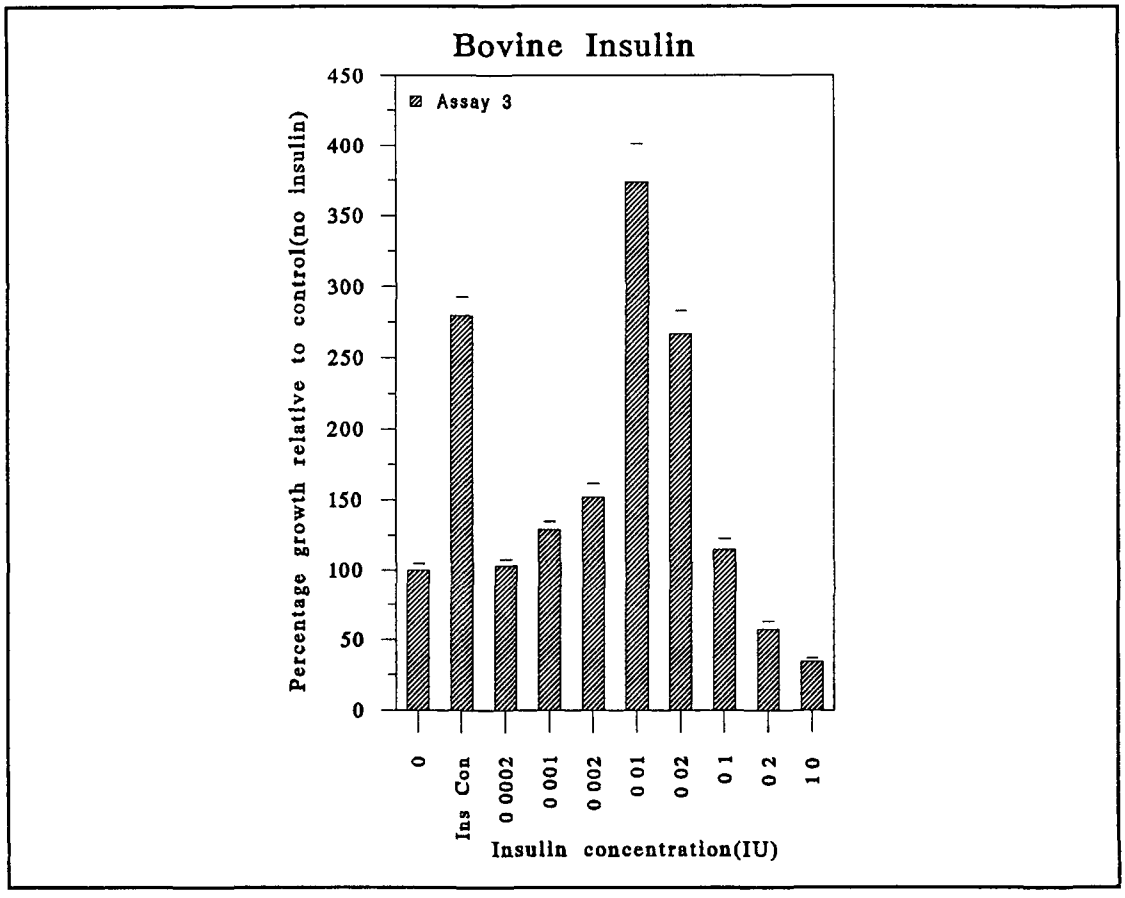


Figure 3.4.2.1 shows the growth response of CHOK1 cells to bovine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 2 1

Table 3 4 2 1 Growth response to bovine insulin

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4	ASSAY 5
IU/ml	µg/ml					
0 0	0 0	100 0 ± 4 26	100 0 ± 9 44	100 0 ± 4 18	100 0 ± 3 33	100 0 ± 9 72
Ins Con	Ins Con	376 5 ± 19 8	261 7 ± 21 1	279 7 ± 12 9	303 3 ± 25 5	564 6 ± 55 5
0 0002 IU	0 00778	172 4 ± 26 3	149 5 ± 14 9	103 1 ± 4 79	104 0 ± 6 66	126 5 ± 9 43
0 001 IU	0 0389	250 0 ± 25 4	242 2 ± 22 2	128 9 ± 5 91	145 5 ± 14 4	149 5 ± 19 5
0 002 IU	0 0778	392 0 ± 27 7	345 0 ± 18 9	151 9 ± 9 68	185 6 ± 22 8	303 1 ± 57 2
0 01 IU	0 389	508 1 ± 45 9	356 8 ± 30 9	373 5 ± 28 1	307 8 ± 25 5	664 2 ± 28 2
0 02 IU	0 778	450 6 ± 16 3	318 9 ± 23 9	266 5 ± 16 5	296 3 ± 24 2	663 0 ± 36 2
0 1 IU	3 89	373 2 ± 26 2	264 4 ± 33 3	114 9 ± 7 767	171 6 ± 12 1	383 3 ± 29 5
0 2 IU	7 78	375 6 ± 23 8	260 0 ± 30 5	57 21 ± 5 71	142 0 ± 8 50	344 3 ± 40 1
1 0 IU	38 7	355 8 ± 25 6	306 0 ± 17 8	34 16 ± 2 47	62 22 ± 1 11	38 05 ± 3 00

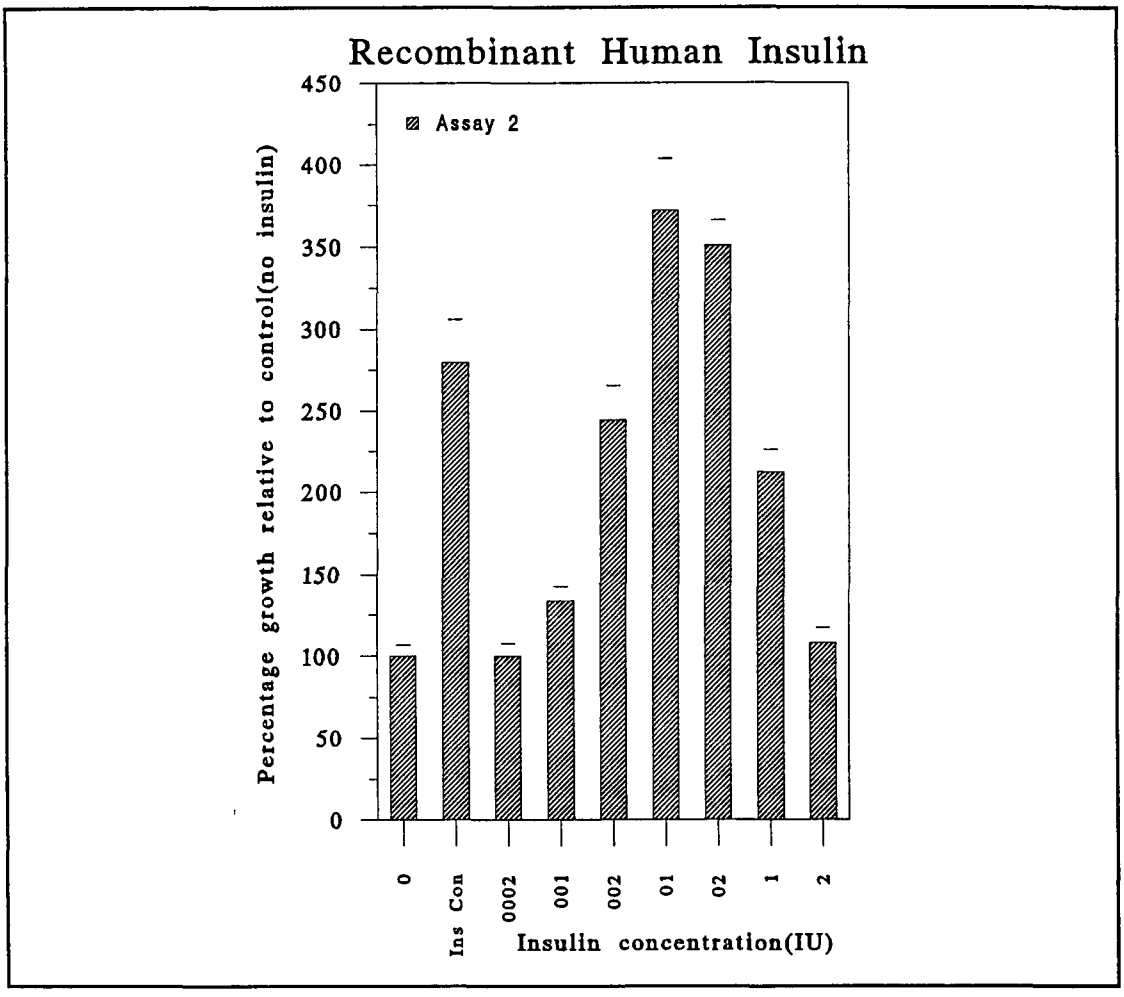


Figure 3.4.2.2 shows the growth response of CHOK1 cells to recombinant human insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 2

Table 3 4 2 2 Growth response to recombinant human insulin

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4	ASSAY 5
IU/ml	μg/ml					
0 0	0 0	100 0 ± 6 86	100 0 ± 7 04	100 0 ± 7 48	100 0 ± 7 29	100 0 ± 5 82
Ins Con	Ins Con	279 9 ± 25 8	392 3 ± 23 2	267 0 ± 19 3	326 9 ± 25 3	659 1 ± 47 6
0 0002 IU	0 00706	100 0 ± 7 32	120 3 ± 13 2	117 2 ± 7 36	98 46 ± 7 27	125 2 ± 10 6
0 001 IU	0 0353	134 1 ± 8 78	233 1 ± 24 1	123 7 ± 12 6	97 69 ± 7 29	141 2 ± 10 8
0 002 IU	0 0706	244 4 ± 20 9	348 2 ± 19 1	123 7 ± 12 3	110 9 ± 9 19	154 7 ± 12 9
0 01 IU	0 353	372 2 ± 31 2	487 4 ± 37 4	330 9 ± 27 9	179 1 ± 19 1	195 5 ± 33 8
0 02 IU	0 706	350 9 ± 15 3	502 6 ± 23 2	298 0 ± 14 4	304 1 ± 24 9	613 2 ± 61 2
0 1 IU	3 53	211 9 ± 14 1	352 9 ± 27 2	160 9 ± 12 0	231 1 ± 23 0	647 8 ± 51 2
0 2 IU	7 06	108 0 ± 9 27	140 5 ± 7 17	81 51 ± 3 99	190 6 ± 12 6	648 7 ± 21 0

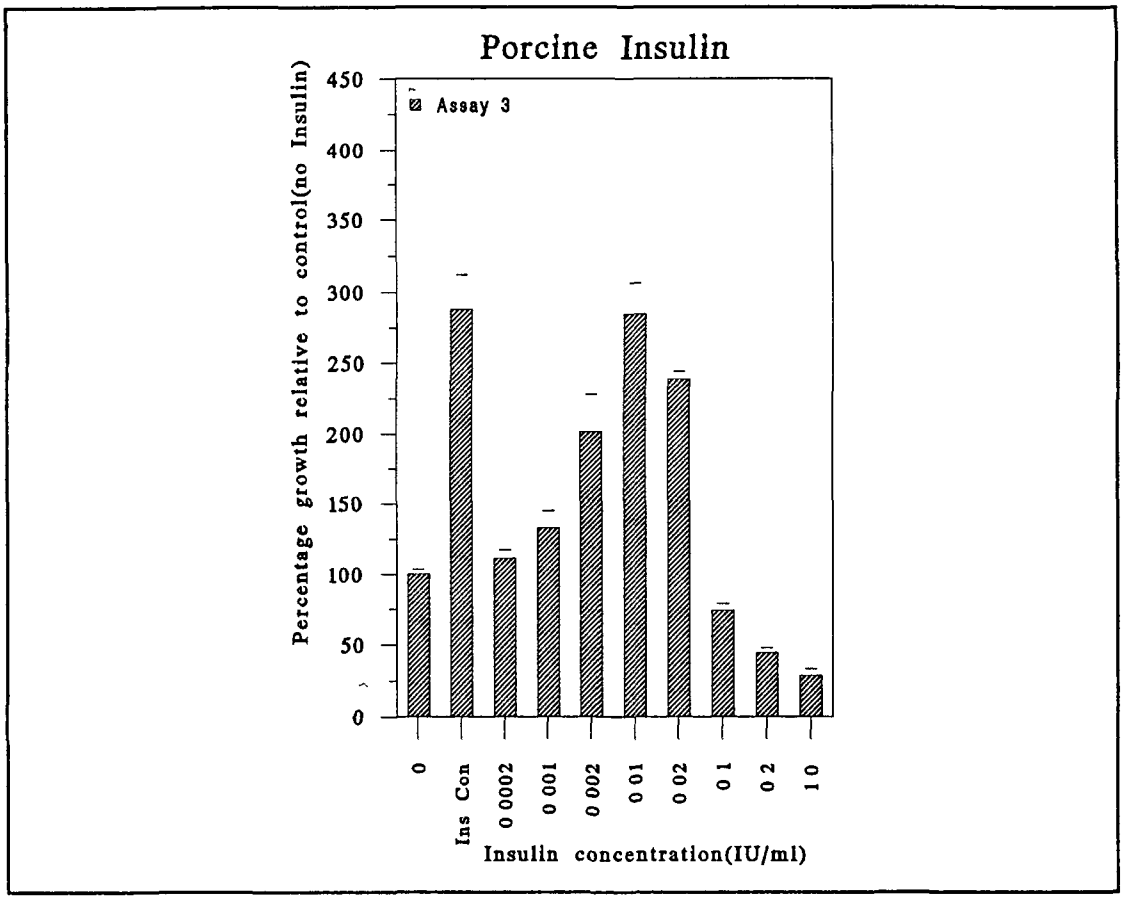


Figure 3.4.2.3 shows the growth response of CHOK1 cells to porcine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 2 3

Table 3 4 2 3 Growth response to porcine insulin

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4	ASSAY 5
IU/ml	μg/ml					
0 0	0 0	100 0 ± 4 33	100 0 ± 7 04	100 0 ± 3 42	100 0 ± 6 71	100 0 ± 3 69
Ins Con	Ins Con	308 8 ± 12 0	355 9 ± 23 2	287 7 ± 19 3	300 9 ± 22 4	585 5 ± 29 3
0 0002	0 00766	202 5 ± 20 2	125 7 ± 13 2	111 3 ± 7 36	101 9 ± 6 71	133 1 ± 13 2
0 001	0 0383	395 1 ± 23 8	259 7 ± 24 1	312 6 ± 12 6	125 2 ± 13 1	176 1 ± 13 0
0 002	0 0766	387 6 ± 30 7	358 2 ± 19 1	201 4 ± 12 3	172 4 ± 14 5	376 0 ± 29 8
0 01	0 383	374 8 ± 30 4	416 6 ± 37 4	284 5 ± 27 9	291 1 ± 20 3	671 1 ± 29 7
0 02	0 766	266 8 ± 9 41	411 1 ± 23 2	238 5 ± 14 4	278 5 ± 27 1	569 4 ± 25 9
0 1	3 83	63 39 ± 4 05	201 5 ± 27 2	74 48 ± 12 0	152 0 ± 13 5	422 0 ± 41 7
0 2	7 66	40 28 ± 1 02	73 21 ± 9 97	44 48 ± 3 14	138 3 ± 10 3	306 6 ± 45 9
0 2	38 3	39 77 ± 2 04	37 41 ± 1 70	28 75 ± 4 49	57 94 ± 0 93	48 05 ± 6 55

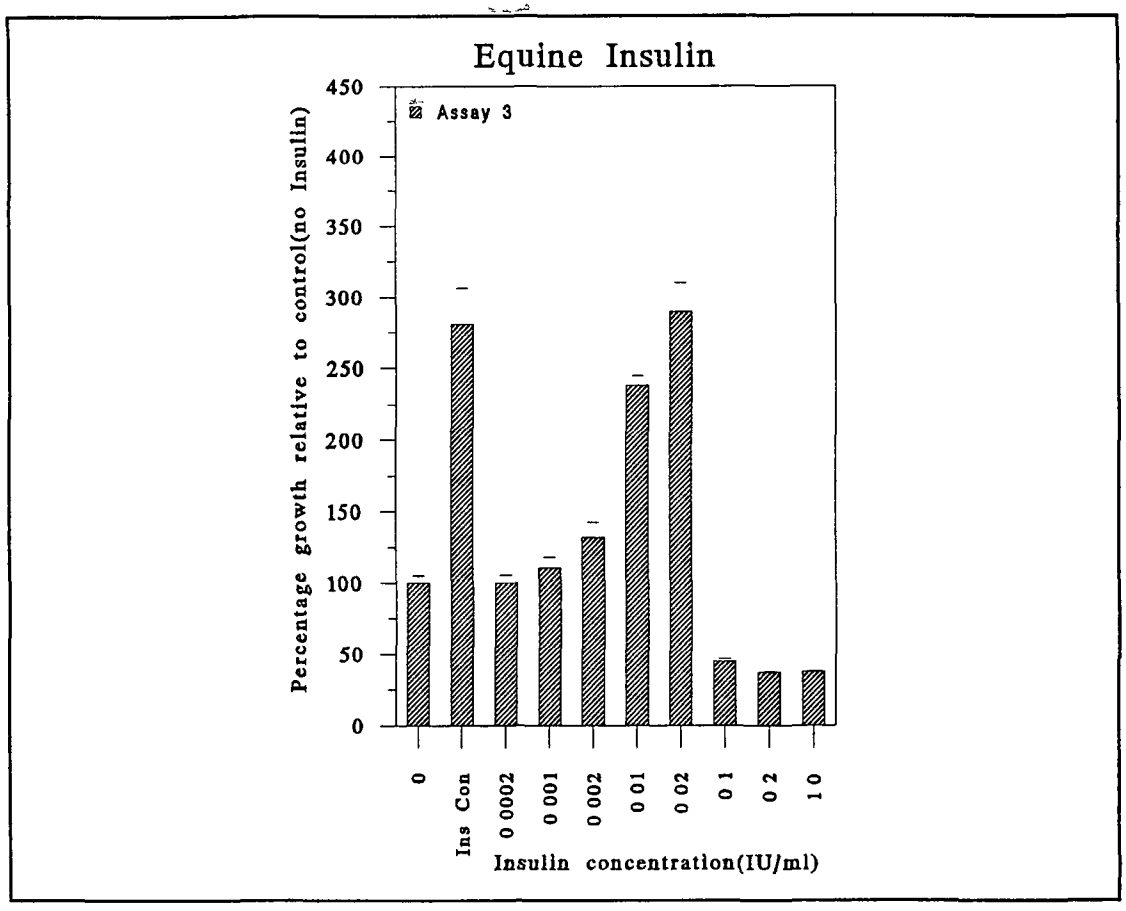


Figure 3.4.2.4 shows the growth response of CHOK1 cells to equine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 2 4

Table 3 4 2 4 Growth response to equine insulin

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4	ASSAY 5
IU/ml	μg/ml					
0 0 IU	0 0μg/ml	100 0 ± 6 83	100 0 ± 5 18	100 0 ± 2 81	100 0 ± 2 03	100 0 ± 7 18
Ins Con	Ins Con	384 5 ± 18 9	280 6 ± 25 5	294 4 ± 19 9	296 7 ± 28 9	501 9 ± 33 4
0 0002	0 0085	128 2 ± 10 4	100 0 ± 5 52	97 29 ± 3 79	98 88 ± 5 55	108 1 ± 6 83
0 001	0 0425	116 6 ± 12 2	110 6 ± 7 82	105 3 ± 6 32	102 2 ± 3 33	136 8 ± 11 8
0 002	0 085	128 2 ± 3 66	132 0 ± 6 44	107 8 ± 3 37	113 3 ± 5 55	154 0 ± 8 72
0 01	0 425	145 4 ± 11 4	238 1 ± 6 41	128 5 ± 11 2	135 7 ± 13 9	287 2 ± 29 3
0 02	0 850	385 1 ± 36 0	289 8 ± 20 3	201 5 ± 23 2	241 7 ± 17 0	472 5 ± 63 1
0 1	4 25	75 78 ± 8 95	44 75 ± 2 21	118 6 ± 14 27	259 3 ± 21 8	721 3 ± 44 5
0 2	8 5	42 90 ± 2 08	37 01 ± 0 55	48 93 ± 3 49	221 1 ± 21 9	583 9 ± 38 4
1 0	42 50	42 11 ± 1 22	37 57 ± 0 55	31 59 ± 1 26	97 46 ± 10 4	279 1 ± 26 5

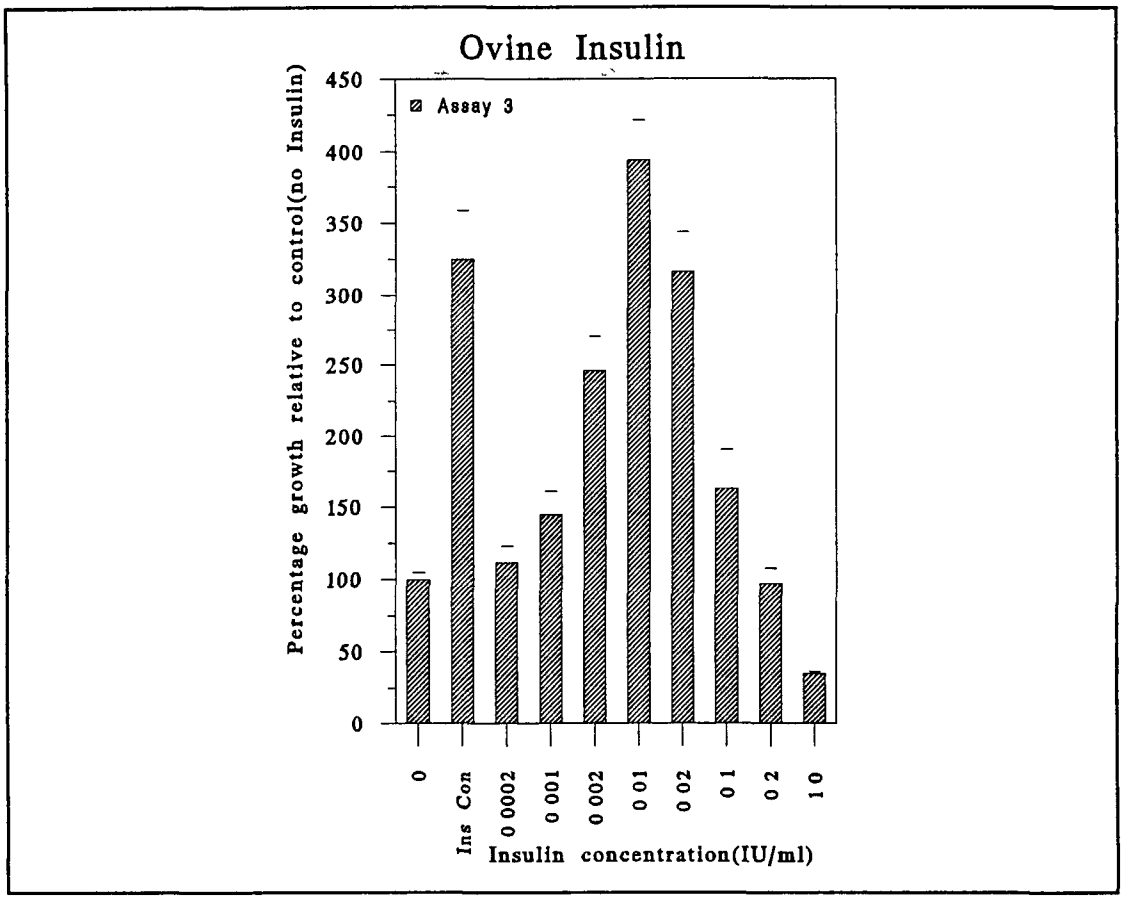


Figure 3.4.2.5 shows the growth response of CHOK1 cells to ovine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 5 separate experiments are shown in Table 3 4 2 5

Table 3 4 2 5 Growth response to ovine insulin

VARIABLES		ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4	ASSAY 5
IU/ml	μg/ml					
0 0	0 0	100 0 ± 9 37	100 0 ± 8 19	100 0 ± 4 92	100 0 ± 4 71	100 0 ± 7 74
Ins Con	Ins Con	363 7 ± 22 8	304 5 ± 17 8	325 1 ± 33 3	331 0 ± 31 9	553 8 ± 27 1
0 0002	0 0086	213 3 ± 0 97	152 2 ± 7 44	145 1 ± 15 9	100 0 ± 4 79	113 0 ± 13 2
0 001	0 0431	242 2 ± 23 5	322 7 ± 33 9	111 7 ± 11 6	111 7 ± 5 32	114 3 ± 6 81
0 002	0 0862	324 5 ± 37 9	441 3 ± 43 9	245 9 ± 24 5	128 7 ± 12 8	152 8 ± 21 5
0 01	0 431	470 8 ± 25 8	432 5 ± 20 4	394 3 ± 27 5	364 9 ± 22 3	535 1 ± 64 8
0 02	0 862	455 0 ± 33 6	385 6 ± 24 0	316 2 ± 27 5	339 2 ± 11 2	608 8 ± 42 2
0 1	4 31	215 5 ± 24 6	133 2 ± 10 3	162 9 ± 27 4	190 1 ± 12 7	405 2 ± 19 7
0 2	8 62	81 87 ± 4 68	59 21 ± 4 63	96 99 ± 10 4	150 6 ± 21 0	290 5 ± 18 2
1 0	43 1	40 93 ± 1 75	43 86 ± 1 17	34 97 ± 1 09	62 76 ± 4 79	51 99 ± 2 23

3.4.3 COMPARISON OF INSULIN STOCKS

In order to investigate the discrepancy between the activity seen with the bovine insulin control and bovine insulin assayed, new stocks of bovine insulin (I4011 and I1882) were obtained The activity of the new and old stocks were compared to determine if the loss of activity was due to a deterioration in the activity of the older stocks

The results are shown Figure 3 4 3 1 and Table 3 4 3 1 In these experiments the extent of stimulation by all stocks of bovine insulin were much higher than that observed in section 3 4 1 (as much as a 3-fold increase) Both new stocks and old stocks showed good stimulation with the newer stocks being only slightly more stimulatory

Looking at a serial dilution of the old stock, the activity reached a maximum stimulation of 8 8-fold to 9 7-fold over the control (no insulin) for three separate repeats Above this concentration, the activity was lower, ranging between 7 1-fold and 8 3-fold stimulation at concentrations of 5 to 10μg/ml

With the new stocks of insulin, a similar trend was seen Maximum stimulation of 9 6-fold to 11-fold and 9 6-fold to 12-fold was seen for I4011 and I1882 respectively at 1μg/ml Thereafter, the activity decreased at 5μg/ml and increased slightly again at 10μg/ml

These results show that maximum stimulation occurred at 1μg/ml in the concentration range tested Inhibition was not seen at 5 to 10μg/ml as in section 3 4 1, but the extent of stimulation appeared to decrease slightly Thus the inhibition seen in section 3 4 1 was not due to the insulin stock Why there was inhibition in this section is not known It may have been due to some inhibitory factor being incorporated into the 10X stocks on initial dilution That the bovine and human stocks did not appear inhibitory at higher concentrations in later experiments could indicate this to be the case

However, the extent of stimulation between the two sets of experiments was quite distinct In this section, CHOK1 cells of passage 6 were used while in section 3 4 1, passage 11 - 13 cells were used The extent of stimulation in section 3 4 3 may have been due to the use of a lower passage number or it could have been due to changes in the serum-free medium composition (*i e* if vitamins or other unstable compounds lost their ability to function) As a result, cells of two different passage numbers were compared to see if passage number could explain the variations in stimulation The results are shown in Figure 3 4 3 2

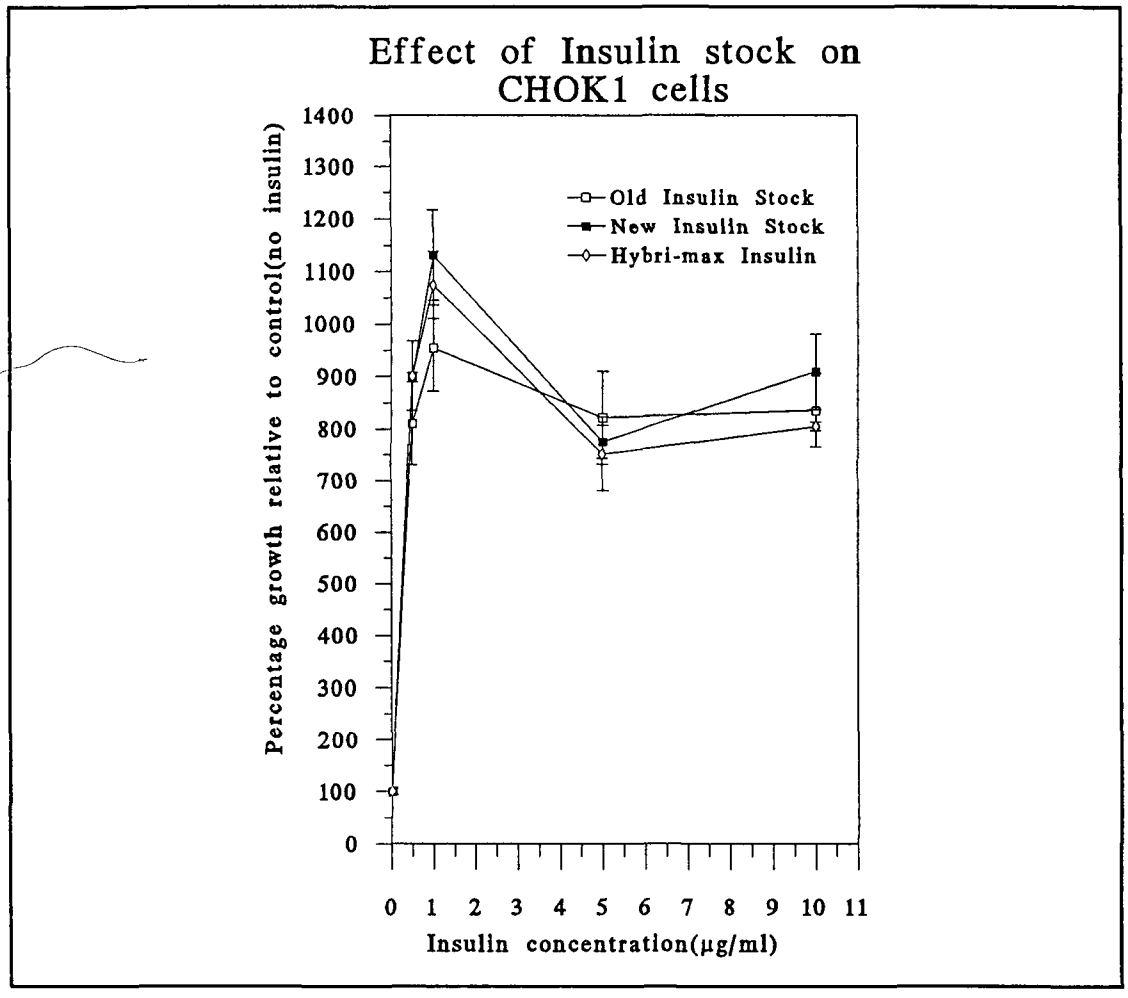


Figure 3.4.3.1 shows the growth response of CHOK1 cells to old and new stocks of bovine insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Tables 3 4 3 1a to c

Table 3 4 3 1a Old insulin stock

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0µg/ml	100 0 ± 10 2	100 0 ± 10 4	100 0 ± 5 50
Ins Con	1152 0 ± 40 4	1257 9 ± 59 6	1054 0 ± 46 7
0 5µg/ml	825 9 ± 63 9	804 9 ± 79 0	810 1 ± 79 0
1 0µg/ml	967 7 ± 75 6	879 2 ± 67 5	953 4 ± 82 5
5 0µg/ml	788 4 ± 67 8	733 8 ± 49 3	820 7 ± 88 6
10 0µg/ml	714 5 ± 58 5	735 0 ± 67 5	834 6 ± 70 4

Table 3.4.3.1b New Insulin stock (I1882)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 μg/ml	100 0 ± 8 71	100 0 ± 9 72	100 0 ± 7 17
Ins Con	1015 4 ± 102	1376 1 ± 128	1162 5 ± 48 1
0 5 μg/ml	795 9 ± 60 9	964 9 ± 107	898 2 ± 87 2
1 0 μg/ml	962 6 ± 78 1	1119 2 ± 75 5	1131 ± 85 9
5 0 μg/ml	755 0 ± 81 3	922 5 ± 94 3	774 6 ± 31 7
10 0 μg/ml	847 1 ± 78 0	1080 6 ± 73 1	908 5 ± 71 9

Table 3.4.3.1c New Insulin stock (I4011)

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0 μg/ml	100 0 ± 8 71	100 0 ± 9 72	100 0 ± 7 17
Ins Con	1015 4 ± 102	1376 1 ± 128	1162 5 ± 48 1
0 5 μg/ml	810 6 ± 72 4	860 6 ± 30 1	900 4 ± 66 0
1 0 μg/ml	966 7 ± 98 7	1223 ± 141	1074 ± 64 4
5 0 μg/ml	818 7 ± 89 4	879 0 ± 32 9	751 0 ± 71 1
10 0 μg/ml	784 5 ± 59 3	1256 ± 87 0	804 0 ± 77 9

For earlier passage cells (passage 4), maximum stimulation of 8 9-fold to 9 7-fold over the control occurred at 1μg/ml with some loss in stimulation at 5 to 10μg/ml For later passage cells (passage 14), maximum stimulation of 11-fold to 17-fold stimulation occurred at 1 - 5μg/ml, with little loss of activity at 10μg/ml These results would suggest that at the higher passage number, the cells had been stimulated to a greater extent by the insulin, or were more dependent on insulin Indeed, the insulin controls for the later passage showed a higher stimulation relative to the control than the earlier passage cells However, this does not account for the change in stimulation seen between sections 3 4 1 and 3 4 2 This would suggest that some factor or factors other than insulin were important for regulating the response of CHOK1 cells to insulin Different stocks of basal media were used in the two sections As Mendiaz recommended, the additional trace elements had been incorporated into the serum-free medium to ensure batch to batch consistency It may be that some of the other elements became deactivated

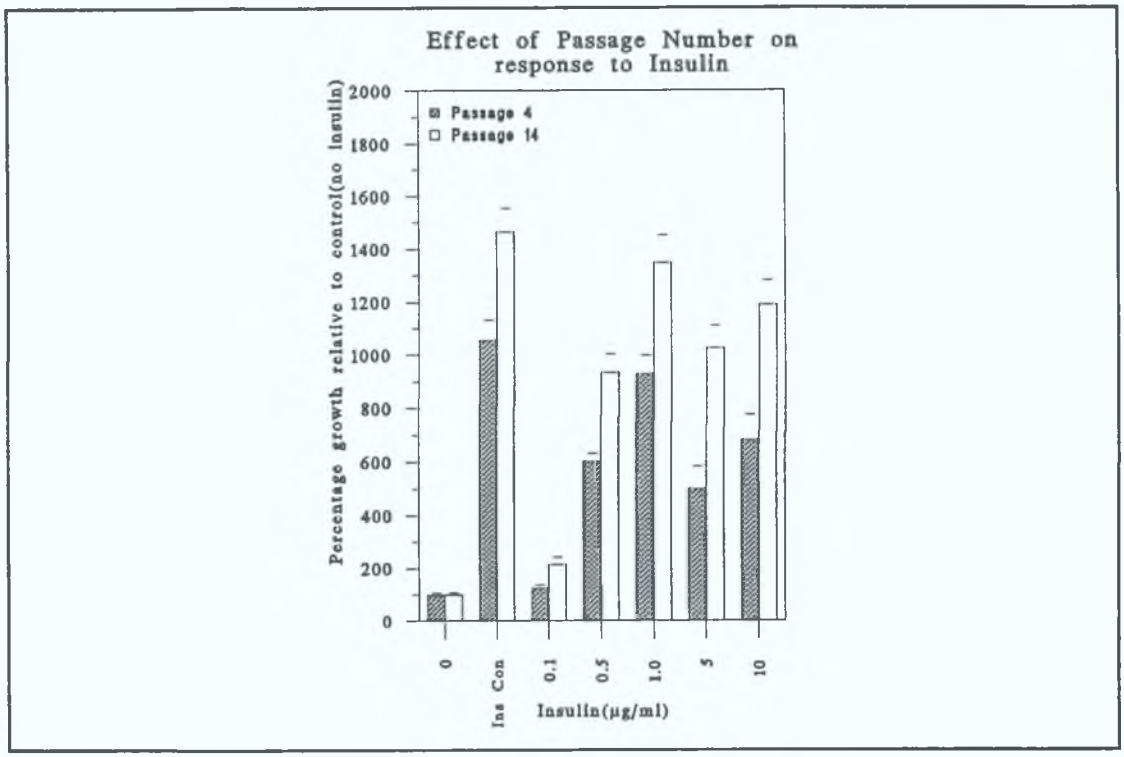


Figure 3.4.3.2 shows the growth response of CHOK1 cells to bovine insulin under serum-free conditions. The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8). The results shown are taken from Assay 3. Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3.4.3.2.

Table 3.4.3.2 Effect of passage number on growth response of CHOK1 cells to insulin

Passage No.	Insulin	ASSAY 1	ASSAY 2	ASSAY 3
4	0.0µg/ml	100.0 ± 9.04	100.0 ± 7.38	100.0 ± 7.94
	Ins Con	978.7 ± 116	1017.5 ± 113	1054.5 ± 77.9
	0.1µg/ml	133.9 ± 9.00	143.7 ± 35.9	124.9 ± 13.0
	0.5µg/ml	503.5 ± 48.9	524.7 ± 69.9	602.4 ± 29.3
	1.0µg/ml	893.3 ± 66.4	972.4 ± 68.2	929.3 ± 68.0
	5.0µg/ml	587.7 ± 53.2	684.6 ± 129	498.7 ± 84.9
	10.0µg/ml	734.4 ± 63.8	629.4 ± 27.6	680.7 ± 95.1
14	0.0µg/ml	100.0 ± 6.79	100.0 ± 8.10	100.0 ± 9.17
	Ins Con	1918.0 ± 63.2	1184.4 ± 79.9	1463.0 ± 91.7
	0.1µg/ml	173.2 ± 17.6	125.9 ± 19.8	215.3 ± 24.9
	0.5µg/ml	1000.0 ± 112.9	780.9 ± 59.0	933.4 ± 69.9
	1.0µg/ml	1701.0 ± 141.7	1013.0 ± 57.0	1348.0 ± 104
	5.0µg/ml	1379.0 ± 150.1	1152.0 ± 52.1	1026.8 ± 82.9
	10.0µg/ml	1547.7 ± 136.1	952.8 ± 3.96	1189.3 ± 91.9

3.4.4 RECOMBINANT HUMAN INSULIN AT HIGHER CONCENTRATIONS

In section 3 4 1, the concentration range tested using the recombinant insulin was lower than that of the bovine insulin In order to see how the activity varied at higher concentrations, a range of 0 0002 to 1 0IU/ml with new stocks was tested The results are shown in Figure 3 4 4

As seen in section 3 4 3, the extent of stimulation by the bovine insulin control was about 3-fold higher than that observed in sections 3 4 1 and 3 4 2 The recombinant insulin was stimulatory in a concentration-dependent manner reaching a maximum stimulation of 10 to 13 8-fold over the control at concentrations of 0 02 - 0 1IU/ml (0 566 - 2 83μg/ml) After this, the stimulation was slightly reduced In 3 of the 5 experiments in section 3 4 2 2, the loss of stimulation at concentrations above 0 02IU/ml was much greater than the loss seen at concentrations of 0 1 to 1IU/inl in 3 4 4

The fact that the stimulation was so high in section 3 4 4, may have been due to a greater dependence on insulin Maximal stimulation occurred between 0 01 - 0 IIU/ml in section 3 4 2 2 and between 0 02 - 0 1IU/ml in section 3 4 4 For the recombinant insulin in section 3 4 4, the ratios of maximum activity compared to the insulin control were 1 350, 1 152 and 1 016 (average and standard deviation of 1 173 $\pm$ 0 168) The average ratio compares favourably to the ratio obtained for the recombinant insulin in section 3 4 2 2 (1 153 $\pm$ 0 183) The ratios of maximum activity versus the insulin control, showed that the activity of both insulins had not changed relative to each other This meant that the change in the extent of stimulation was due to some factor(s) other than insulin

These results show that the activity of the recombinant insulin reaches maximum activity between 0 01 - 0 1IU/ml from both sections and the maximum stimulation was as good if not better than the bovme insulin

The results show that the IGFs are not a suitable replacement for the CHOK1 cells Comparison of the various sources of insulin show that recombinant human insulin can be used as a replacement for bovine-derived insulin The results show changes in the maximum stimulatory concentration of insulin and a difference in morphology to that observed by Mendiaz

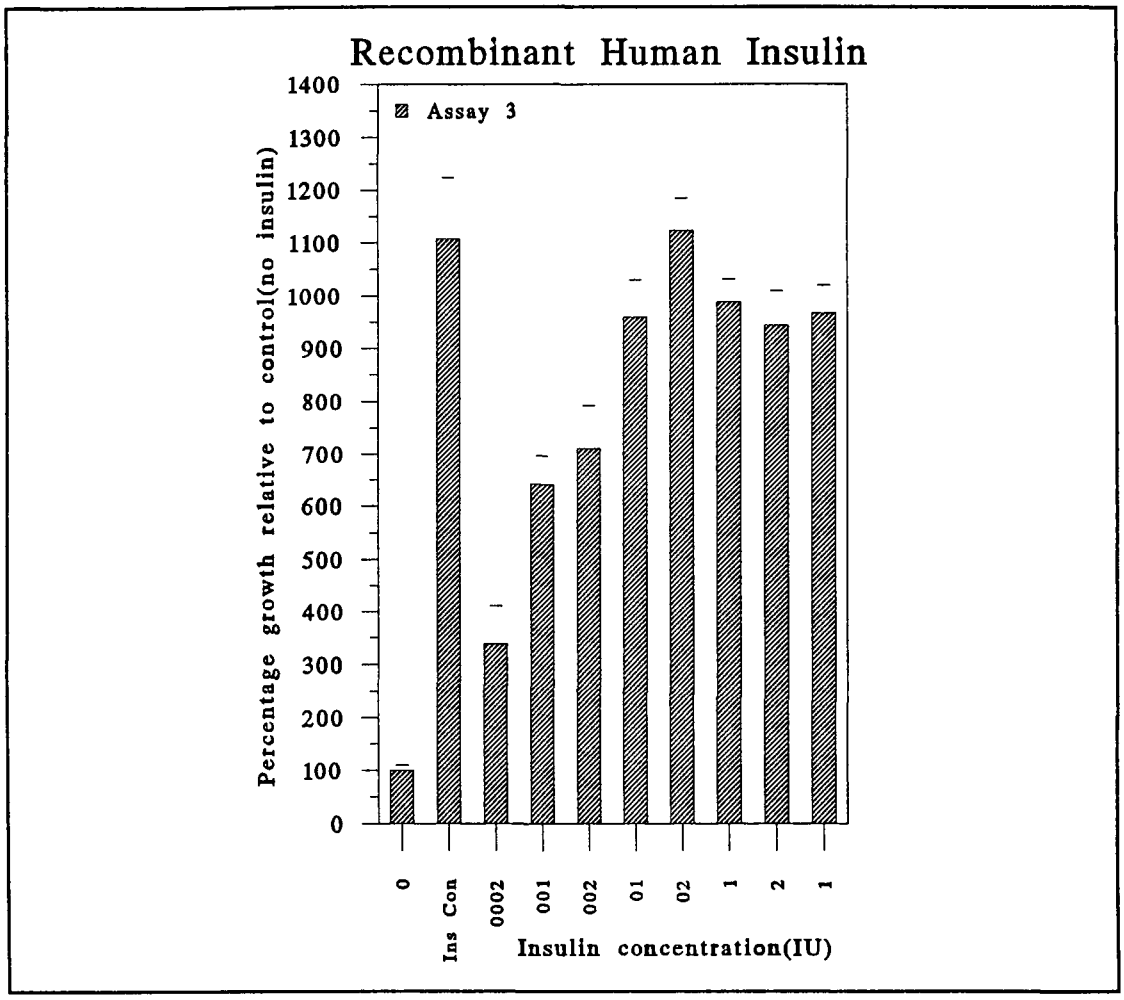


Figure 3.4.4 shows the growth response of CHOK1 cells to recombinant human insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Table 3 4 4

Table 3 4 4 Growth response to recombinant human insulin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0IU/ml	100 0 ± 5 10	100 0 ± 21 4	100 0 ± 9 81
Ins Con	1050 0 ± 49 9	955 5 ± 115	1107 4 ± 116
0 0002IU/ml	278 3 ± 21 1	202 9 ± 11 4	337 6 ± 73 6
0 001IU/ml	400 0 ± 55 6	453 7 ± 44 5	641 4 ± 54 7
0 002IU/ml	656 0 ± 75 0	675 0 ± 51 5	711 8 ± 80 2
0 01IU/ml	840 4 ± 70 0	877 3 ± 144	959 6 ± 70 8
0 02IU/ml	958 6 ± 44 2	1085 5 ± 75 9	1124 6 ± 59 8
0 1IU/ml	1382 5 ± 62 0	815 6 ± 70 2	988 4 ± 43 9
0 2IU/ml	1345 0 ± 127	911 8 ± 157	943 5 ± 65 8
1 0IU/ml	1094 6 ± 64 6	853 5 ± 101	967 6 ± 52 6

3.5.1 EFFECT OF ALBUMINS ON THE GROWTH OF NRK CELLS

BSA has been found to be stimulatory for many cell lines (Nilausen, 1978, Kan and Yamane, 1982, Barlian *et al* , 1993) Due to the 'stickiness' of BSA, it can carry a wide variety of contaminating factors The activity of the albumin is most often related to factors which the albumin carries rather than the albumin molecule itself Fatty acids are most commonly cited as the source of the activity The results outlined in section 3 1 show that bovine serum albumin (BSA) fraction V was stimulatory for NRK cells at concentrations in the range of 0 5 to 10mg/ml with a 2% Donor Horse Serum (DHS) background In order to determine if the activity was due to the presence of fatty acids, a comparison of human and bovine fraction V and fatty acid free albumins was made

This section describes a number of investigations which were carried out to try and determine the cause of the stimulatory activity associated with BSA for NRK cells in low serum-supplemented medium

3.5.1.1.1 Bovine Serum Albumin - Fraction V

The growth response of NRK cells to BSA fraction V (Sigma A4919, lot 110H - 04635) is shown in Figure 3 5 1 1 1 and Tables 3 5 1 1 1a and b Two independent experiments showed that increasing the albumin concentration resulted in increased stimulation within the concentration range tested The extent of stimulation varied between the two experiments With dye elution or acid phosphatase as the end point, stimulation varied between 2-fold and 2 5-fold stimulation over the control (2% DHS), while for image analysis, the effect of BSA fraction V was much greater, with a 2-fold to 10-fold stimulation over the control at 2 5 - 5 0mg/ml

Table 3.5 1 1 1 a Effect of BSA - fraction V (mg/ml) on NRK cells

ASSAY 1	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 13 8	100 0 (110 4, 89 40)	100 0 ± 3 51	100 0 ± 8 46
+ 0 5 A	106 0 ± 15 7	110 0 (123 2, 99 10)	176 6 ± 3 76	707 0 ± 28 8
+ 1 0 A	120 0 ± 1 63	122 0 (146 9, 96 70)	185 2 ± 2 65	724 0 ± 64 1
+ 2 5 A	132 0 ± 11 6	118 0 (133 6, 103 0)	234 8 ± 10 6	1033 ± 60 2
+ 5 0 A	165 0 ± 16 9	150 9 (157 4, 144 5)	246 8 ± 4 32	937 0 ± 2 86
+ 10 0 A	177 0 ± 12 6	149 6 (138 9, 160 4)	214 2 ± 15 7	573 0 ± 48 8
5% DHS	183 3 ± 11 8	186 0 (191 0, 203 2)	189 3 ± 31 3	715 0 ± 50 2

All results except those for cell number are expressed as the average percentage growth relative to the control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=6 for dye elution and n=3 for image analysis) Results for cell number are expressed as the average percentage growth relative to control (2% DHS) As there were only two values for each assay condition, a standard deviation was not carried out Instead, the results of the two separate readings were expressed as the percentage growth relative to average growth obtained with the control, 2% DHS
Abbreviations A = mg/ml albumin, BSA fraction V

The results show similar trends in the two assays for acid phosphatase, cell number and dye elution When image analysis was used as the end point, the extent of stimulation was much greater than that seen with the other end points Yet, when the dye was eluted off the plates that were read on the image analyser, the extent of stimulation was lower
As image analysis measures colony area as a basis of comparison, the results would indicate that the BSA was not only promoting cell growth but was also promoting cell spreading

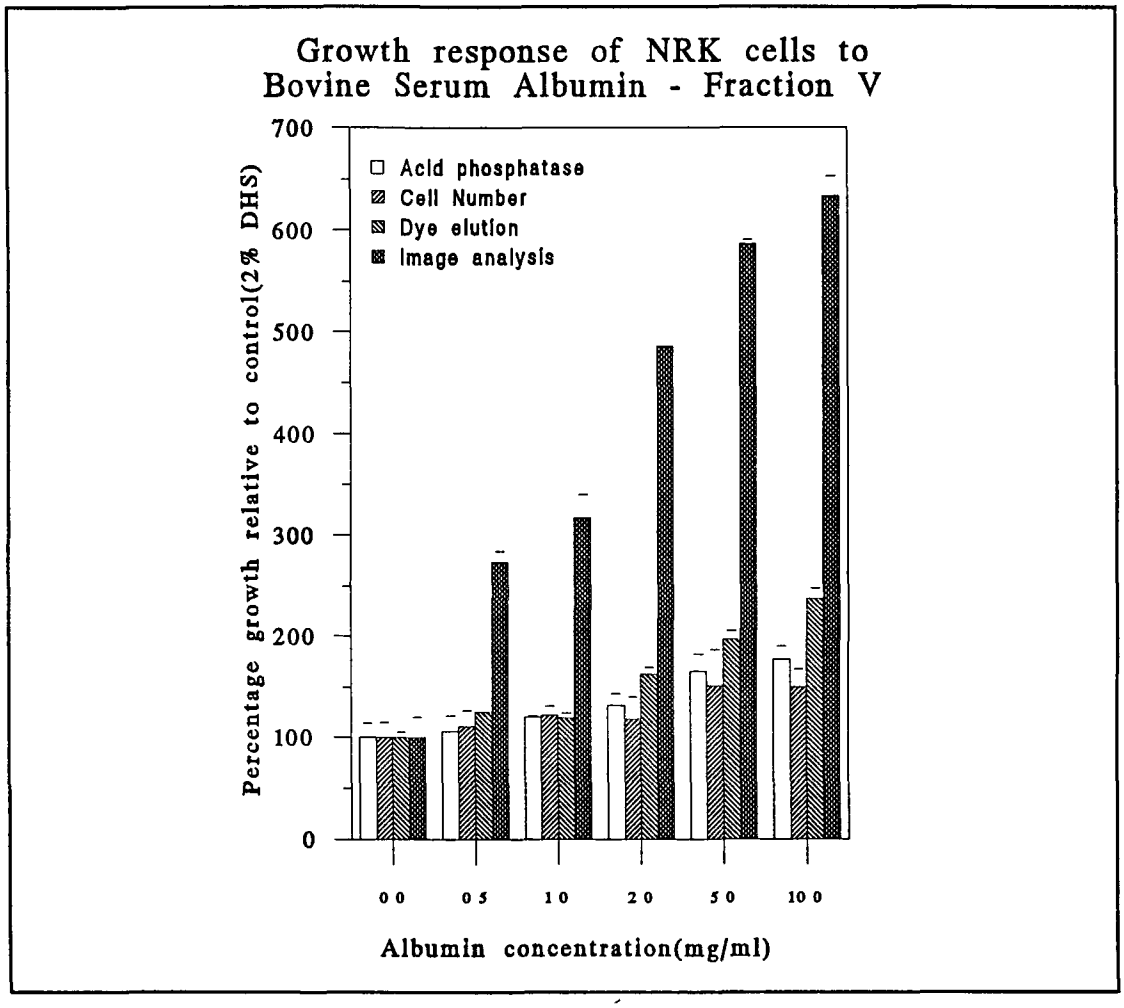


Figure 3.5.1.1.1 shows the growth response of NRK cells to BSA fraction V for Assay 2 Results for all end points except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=6 for dye elution, n=3 for image analysis) For cell number, only one set of values were obtained, so no statistical analysis could be carried out Results were therefore, expressed as the percentage growth relative to the control (2% DHS) Abbreviations A = mg/ml albumin, BSA fraction V Results for separate experiments are shown in Tables 3 5 1 1 1a and b

Table 3 5.1.1.1.b Effect of BSA - fraction V (mg/ml) on NRK cells

ASSAY 2	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 9 52	100 0	100 0 ± 5 13	100 0 ± 20 3
+ 0 5 A	129 8 ± 14 2	133 0	125 0 ± 0 08	274 0 ± 10 9
+ 1 0 A	153 4 ± 11 9	174 0	119 5 ± 5 98	317 0 ± 22 3
+ 2 5 A	140 0 ± 6 60	169 0	162 7 ± 5 96	486 0 ± 0 59
+ 5 0 A	171 0 ± 11 9	181 0	196 8 ± 8 02	586 4 ± 4 36
+ 10 0 A	217 0 ± 16 8	202 0	237 4 ± 10 5	634 0 ± 19 3
5% DHS	190 9 ± 17 4	188 0	257 6 ± 16 6	516 5 ± 13 5

3.5.1.1.2 Human Serum Albumin - fraction V

HSA fraction V (Sigma A1653, lot 86F - 9383) was found to be growth stimulatory with all end points used (Figure 3 5 1 1 2) In the concentration range tested, growth increased with increasing albumin up to 5 - 10mg/ml, above this concentration a plateau was seen with all end points except cell number Stimulation in the first experiment was lower than that obtained with the second and third For assays 2 and 3, image analysis showed a greater level of stimulation, with 300% stimulation relative to control at 5mg/ml, while for the other end points, maximum stimulation was around 200% stimulation relative to control (2% DHS)

When compared to BSA fraction V, both showed similar stimulation with acid phosphatase, cell number and dye elution as end points Image analysis results for HSA fraction V were lower than those obtained with BSA fraction V So although both albumins stimulated growth to a similar extent (acid phosphatase, cell number and dye elution as the end points), BSA fraction V appeared to have a greater effect on cell spreading than HSA fraction V (as seen with image analysis)

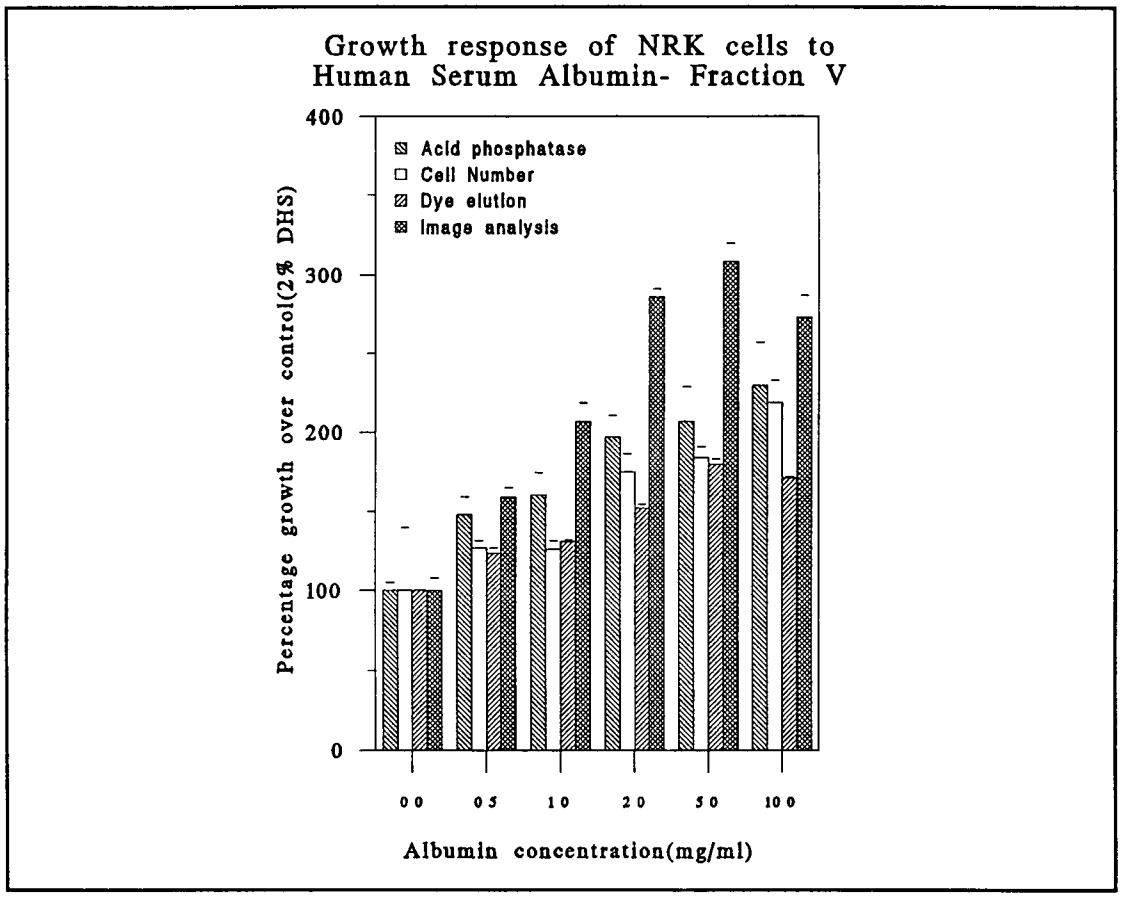


Figure 3.5.1.1.2 shows the growth response of NRK cells to HSA - fraction V for Assay 2 All results except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=6 for dye elution and n=3 for image analysis) For cell number, results are expressed as described Table 3 5 1 1 1a Abbreviations A = mg/ml albumin.

Table 3.5.1.1.2.a Effect of HSA - fraction V (mg/ml) on NRK cells

ASSAY 1	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 4 83	100 0 (96 71, 103,3)	100 0 ± 2 29	100 0 ± 12 2
+ 0 5 A	117 5 ± 8 69	117 8 (117 4, 118 3)	100 5 ± 4 54	163 0 ± 5 46
+ 1 0 A	130 0 ± 9 65	132 0 (120 3, 143 8)	125 2 ± 5 74	171 1 ± 1 36
+ 2 5 A	155 8 ± 12 8	128 6 (113 6, 143 5)	122 5 ± 9 43	199 8 ± 6 45
+ 5 0 A	183 4 ± 4 78	116 6 (115 8, 117 6)	129 9 ± 12 5	223 4 ± 3 14
+ 10 0 A	181 0 ± 12 8	115 5 (127 0, 103 9)	129 8 ± 11 0	215 0 ± 9 25
5% DHS	181 0 ± 11 7	167 2 (183 9, 150 5)	211 0 ± 8 99	332 0 ± 24 2

Table 3.5 1.1.2.b Effect of HSA - fraction V (mg/ml) on NRK cells

ASSAY 2	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 4 96	100 0 (128 2, 71 83)	100 0 ± 0 70	100 0 ± 12 2
+ 0 5 A	148 0 ± 11 1	126 8 (130 0, 123 6)	123 6 ± 3 61	159 0 ± 7 88
+ 1 0 A	160 7 ± 13 7	126 3 (130 0, 122 6)	131 2 ± 1 36	207 0 ± 11 4
+ 2 5 A	197 0 ± 13 7	174 9 (166 6, 183 2)	152 3 ± 2 37	286 4 ± 4 87
+ 5 0 A	207 0 ± 21 8	183 9 (185 6, 182 4)	179 8 ± 3 39	308 6 ± 11 2
+ 10 0 A	229 8 ± 27 3	218 8 (208 8, 228 8)	171 3 ± 0 41	273 0 ± 14 3
5% DHS	174 4 ± 7 61	175 2 (194 9, 155 9)	222 0 ± 10 0	515 0 ± 50 2

Results for all end points except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Results for cell number are as described in Table 3 5 1 1 1a Abbreviations A = mg/ml albumin, HSA - fraction V

Table 3.5 1.1.2.c Effect of HSA - fraction V (mg/ml) on NRK cells

ASSAY 3	ACID PHOSPHATASE	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 9 52	100 0 ± 8 70	100 0 ± 3 43
+ 0 5 A	100 0 ± 14 2	131 1 ± 5 23	131 0 ± 11 3
+ 1 0 A	181 6 ± 11 9	159 4 ± 7 46	299 0 ± 19 1
+ 2 5 A	214 0 ± 6 60	215 1 ± 10 1	234 0 ± 20 5
+ 5 0 A	------	230 6 ± 18 4	346 0 ± 41 0
+ 10 0 A	------	223 2 ± 11 6	257 0 ± 23 3
5% DHS	------	254 2 ± 5 10	661 0 ± 34 7

Results are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Abbreviations A = mg/ml albumin, HSA fraction V

3.5.1.1.3 Bovine Serum Albumin - fatty acid free

BSA fatty acid free (Pentex 82-042-2, lot 303) showed growth stimulation in three separate experiments for all end points except image analysis Results are shown in Figure 3 5 1 1 3 and Tables 3 5 1 1 3a and b The stimulation increased with increasing albumin concentration but was not strictly linear The extent of stimulation varied between experiments In two of three assays using image analysis as the end point, inhibition occurred while in Assay 2 huge stimulation of up to 10-fold was seen For acid phosphatase as the end point, maximum stimulation of 51 to 207% above the control (2% DHS) was seen at 5 - 10mg/ml For cell number and dye elution, the stimulation was lower (37% to 83% stimulatm above the control)

When the dye was eluted from the plates read on an image analyser and the absorbance measured, stimulation was seen in the three assays BSA fatty acid free was not as stimulatory as BSA fraction V While BSA fraction V caused stimulation when image analysis was used as the end point, BSA fatty acid free showed inhibition in Assay 2 It would appear that BSA fatty acid free was not stimulatory to cell spreading but did promote cell growth

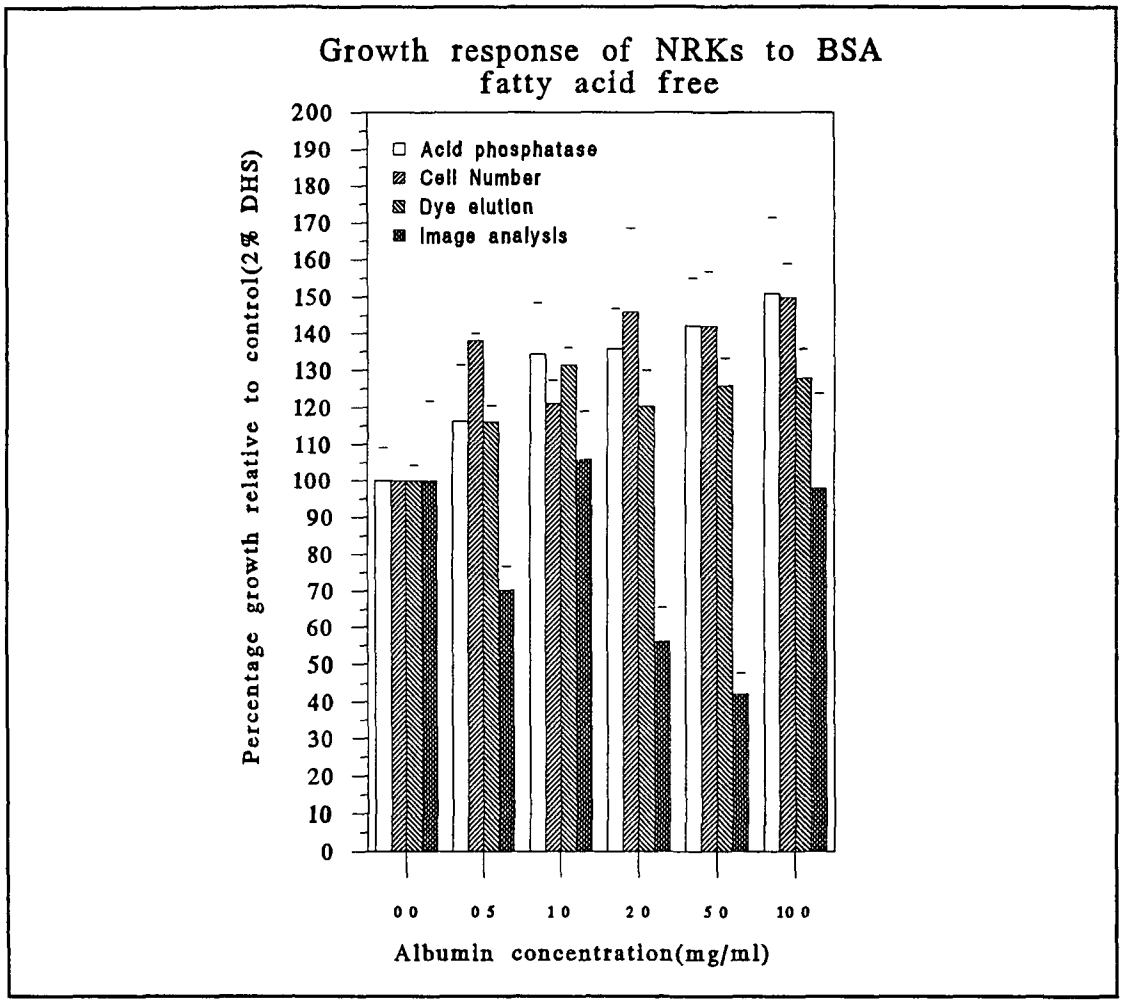


Figure 3.5.1.1.3 shows the growth response of NRK cells to BSA fatty acid free from Assay 1 All results except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=6 for dye elution and n=3 for Image analysis) For cell number, results are expressed as described in Table 3 5 1 1 1a Abbreviations A = mg/ml albumin, BSA fatty acid free

Table 3.5.1.1.3.a Effect of BSA - fatty acid free (mg/ml) on NRK cells

ASSAY 1	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 9 14	100 0 (98 34, 101 6)	100 0 ± 4 25	100 0 ± 21 7
+ 0 5 A	116 2 ± 15 3	137 9 (139 4, 136 4)	116 1 ± 4 49	70 30 ± 6 56
+ 1 0 A	134 3 ± 14 2	121 1 (125 6, 116 5)	131 4 ± 4 75	106 0 ± 13 1
+ 2 5 A	135 7 ± 11 2	145 6 (161 6, 129 7)	120 4 ± 9 65	56 12 ± 9 30
+ 5 0 A	142 0 ± 13 1	141 9 (131 4, 152 2)	126 0 ± 7 20	42 14 ± 5 68
+ 10 0 A	151 0 ± 20 3	150 4 (156 8, 144 1)	127 9 ± 7 90	97 90 ± 25 9
5% DHS	158 0 ± 14 4	239 7 (236 4, 243 0)	241 0 ± 8 02	332 0 ± 24 4

Table 3.5.1.1.3.b Effect of BSA - fatty acid free (mg/ml) on NRK cells

ASSAY 2	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 13 3	100 0 (93 09, 106 9)	100 0 ± 2 18	100 0 ± 8 73
+ 0 5 A	153 9 ± 6 57	117 9 (110 2, 125 5)	114 0 ± 5 06	172 0 ± 14 1
+ 1 0 A	155 5 ± 2 98	133 8 (133 1, 134 5)	117 3 ± 1 86	174 0 ± 36 7
+ 2 5 A	197 0 ± 13 6	125 2 (126 0, 124 3)	138 7 ± 2 03	451 5 ± 16 4
+ 5 0 A	207 0 ± 6 85	137 4 (153 0, 121 9)	160 7 ± 1 98	953 0 ± 97 4
+ 10 0 A	182 1 ± 6 85	121 4 (157 9, 84 90)	140 1 ± 3 28	1015 ± 74 5
5% DHS	----------	---------	220 0 ± 2 90	1610 ± 117

Results for all end points except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Results for cell number are expressed as described in Table 3 5 1 1 1a Abbreviations A = mg/ml albumin, BSA fatty acid free

Table 3 5.1.1.3.c Effect of BSA - fatty acid free (mg/ml) on NRK cells

ASSAY 3	ACID PHOSPHATASE	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 9 37	100 0 ± 4 45	100 0 ± 6 54
+ 0 5 A	107 9 ± 10 2	103 1 ± 8 37	80 22 ± 6 21
+ 1 0 A	124 6 ± 9 09	112 1 ± 2 96	77 97 ± 9 18
+ 2 5 A	135 8 ± 8 39	128 1 ± 1 43	62 72 ± 7 18
+ 5 0 A	156 4 ± 5 67	169 2 ± 6 86	51 12 ± 4 34
+ 10 0 A	145 3 ± 7 63	182 8 ± 8 74	45 05 ± 5 95
5% DHS	190 9 ± 17 4	162 9 ± 5 84	142 4 ± 19 9

Results for all end points except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Abbreviations A = mg/ml albumin, BSA fatty acid free

3.5.1.1.4 Human Serum Albumin - fatty acid free

Human Serum Albumin (HSA) fatty acid free (Sigma A3782, lot 119F9303) was found to be inhibitory on the growth of NRK cells The results for three replicate experiments are shown in Figure 3 5 1 1 4 and Tables 3 5 1 1 4a to c For acid phosphatase, cell number and dye elution, increasing the albumin concentration generally resulted in reduced growth with 15 - 20%, 27 -30% and 40 - 45% inhibition obtained respectively, at the highest concentration tested (10mg/ml) The results obtained from image analysis showed stimulation in two of three assays with inhibition in the third When the crystal violet dye was eluted from the plates which had been read on an image analyser, the same inhibitory trend was seen with dye elution for each experiment However, the extent of inhibition with dye elution was less than that obtained with image analysis

HSA fatty acid free was like BSA fatty acid free (in two of three assays) in that image analysis results showed the two fatty acid free albumins to be inhibitory to NRK cells when colony area was used as the basis of measurement (image analysis) However, while BSA fatty acid free was stimulatory with the rest of the end points used, HSA fatty acid free was inhibitory

Having looked at bovine and human derived albumins, both fraction V and fatty acid free, the order of activity (stimulation to inhibition) was BSA fraction V > HSA fraction V > BSA-faf > HSA-faf The fact that the fraction Vs were better than the fatty acid free albumins, suggest that some active factor was lost during removal of fatty acids Also the bovine- derived albumins were better than the human-derived albumins The difference in response may depend on the source of the albumin and thus what factors it was carrying or it may have been due to batch to batch variability of albumins

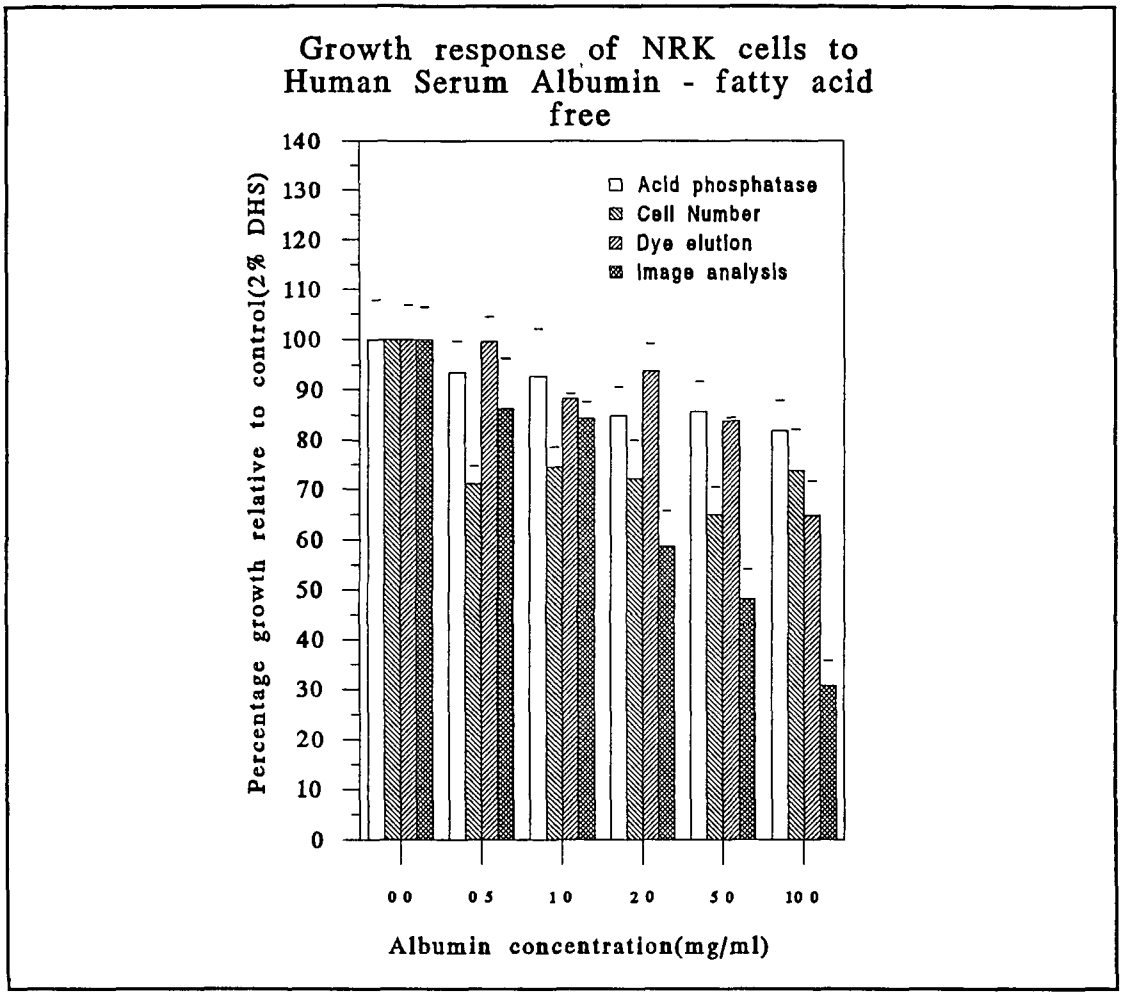


Figure 3.5.1.1.4 shows the growth response of NRK cells to HSA fatty acid free All results except for cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=6 for dye elution and n=3 for image analysis) For cell number, results are expressed as described in Table 3 5 1 1 1a Abbreviations A = mg/ml albumin, HSA fatty acid free

Table 3.5.1 1 4a Effect of HSA-fatty acid free (mg/ml) on NRK cells

ASSAY 1	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 7 84	100 0 (105 6, 94 43)	100 0 ± 6 77	100 0 ± 7 34
+ 0 5 A	93 55 ± 6 11	71 10 (68 80, 73 60)	99 60 ± 4 82	86 25 ± 6 46
+ 1 0 A	92 73 ± 9 40	75 60 (71 50, 79 60)	88 25 ± 1 07	84 24 ± 10 0
+ 2 5 A	84 79 ± 5 78	72 18 (77 70, 66 66)	93 82 ± 5 40	58 74 ± 3 43
+ 5 0 A	85 62 ± 6 07	64 91 (68 85, 60 97)	83 72 ± 0 69	48 20 ± 6 94
+ 10 0 A	81 79 ± 6 05	73 76 (67 88, 79 64)	64 83 ± 6 86	30 66 ± 5 87
5% DHS	168 0 ± 17 5	178 6 (172 0, 185 0)	199 2 ± 4 11	302 0 ± 7 34

Table 3.5 1 1.4b Effect of HSA-fatty acid free (mg/ml) on NRK cells

ASSAY 2	ACID PHOSPHATASE	CELL NUMBER	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 7 14	100 0 (110 1, 89 94)	100 0 ± 4 61	100 0 ± 7 37
+ 0 5 A	109 5 ± 4 78	104 0 (111 9, 96 20)	89 99 ± 2 88	120 0 ± 15 3
+ 1 0 A	99 55 ± 8 48	101 9 (104 7, 99 10)	88 89 ± 3 79	115 0 ± 11 4
+ 2 5 A	103 0 ± 8 03	78 93 (76 10, 81 70)	83 93 ± 2 16	139 0 ± 5 37
+ 5 0 A	88 14 ± 2 01	58 30 (52 83, 63 83)	79 10 ± 4 55	100 0 ± 4 47
+ 10 0 A	85 27 ± 7 14	71 07 (72 30, 69 49)	69 83 ± 5 11	71 70 ± 5 34
5% DHS	178 0 ± 24 1	262 0 (251 2, 272 9)	216 0 ± 4 73	402 0 ± 24 7

Results for all end points except cell number are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Results for cell number are expressed as described in Table 3 5 1 1 1a Abbreviations A = albumin (in this case HSA - fatty acid free)

Table 3.5.1.1.4c Effect of HSA-fatty acid free (mg/ml) on NRK cells

ASSAY 3	ACID PHOSPHATASE	DYE ELUTION	IMAGE ANALYSIS
2% DHS	100 0 ± 5 00	100 0 ± 4 32	100 0 ± 10 8
+ 0 5 A	87 75 ± 2 52	77 74 ± 6 81	88 04 ± 5 59
+ 1 0 A	85 22 ± 6 30	75 07 ± 2 44	147 5 ± 14 5
+ 2 5 A	85 22 ± 4 14	79 56 ± 5 27	148 9 ± 15 9
+ 5 0 A	77 29 ± 5 04	77 06 ± 5 39	334 4 ± 26 9
+ 10 0 A	69 55 ± 4 86	71 15 ± 5 66	379 5 ± 6 48
5% DHS	125 4 ± 12 0	146 6 ± 12 8	246 2 ± 22 5

Results for all end points are expressed as the average percentage growth relative to control (2% DHS) ± standard deviation (n=8 for acid phosphatase, n=3 for image analysis and n=6 for dye elution) Abbreviations A = mg/ml albumin, HSA - fatty acid free

3.5.1.2 EFFECT OF ALBUMIN ON SCC-9 CELLS

SCC-9 is a human squamous carcinoma of the tongue which had previously shown a growth response to albumin (Eunan MaGlinchy, Masters thesis in process of submission) The effects of the bovine and human albumins used in section 3 5 1 on the growth of SCC-9 cells were compared

Experiments were set up slightly differently to those for NRK cells The SCC-9 cells were set up with a 0 25% background of FCS in 96-well plates The concentration range of albumin tested was 20 - 1000μg/ml as this was the concentration range in which activity was observed for SCC-9 cells After 7 days' incubation at 37°C and 5% CO_2, the assays were read using acid phosphatase as the end point The results are shown in Figures 3 5 1 2 1 to 4

The results are shown in Figures 3 5 1 2 1 to 4 Unlike the NRK cells (section 3 5 1 1), the fatty acid free (faf) albumins gave the best stimulation Of the fatty acid free albumins, BSA faf showed the best stimulation of all the albumins tested on SCC-9 cells At 500μg/ml an optimum stimulation was seen with growth between 2-fold and 3 5-fold stimulation over control

HSA fraction V (Figure 3 5 1 2 2) showed similar stimulation to that seen with HSA - fatty acid free (Figure 3 5 1 2 4) with a maximum stimulation of 2-fold over the control (0 25% FCS) BSA - fraction V was least stimulatory with a maximum stimulation of 60% over control at 100 - 500μg/ml

For SCC-9 cells, the order of stimulatory activity for the albumins was different to that obtained by NRK cells The order was BSA-faf > HSA fraction V = HSA-faf > BSA fraction V

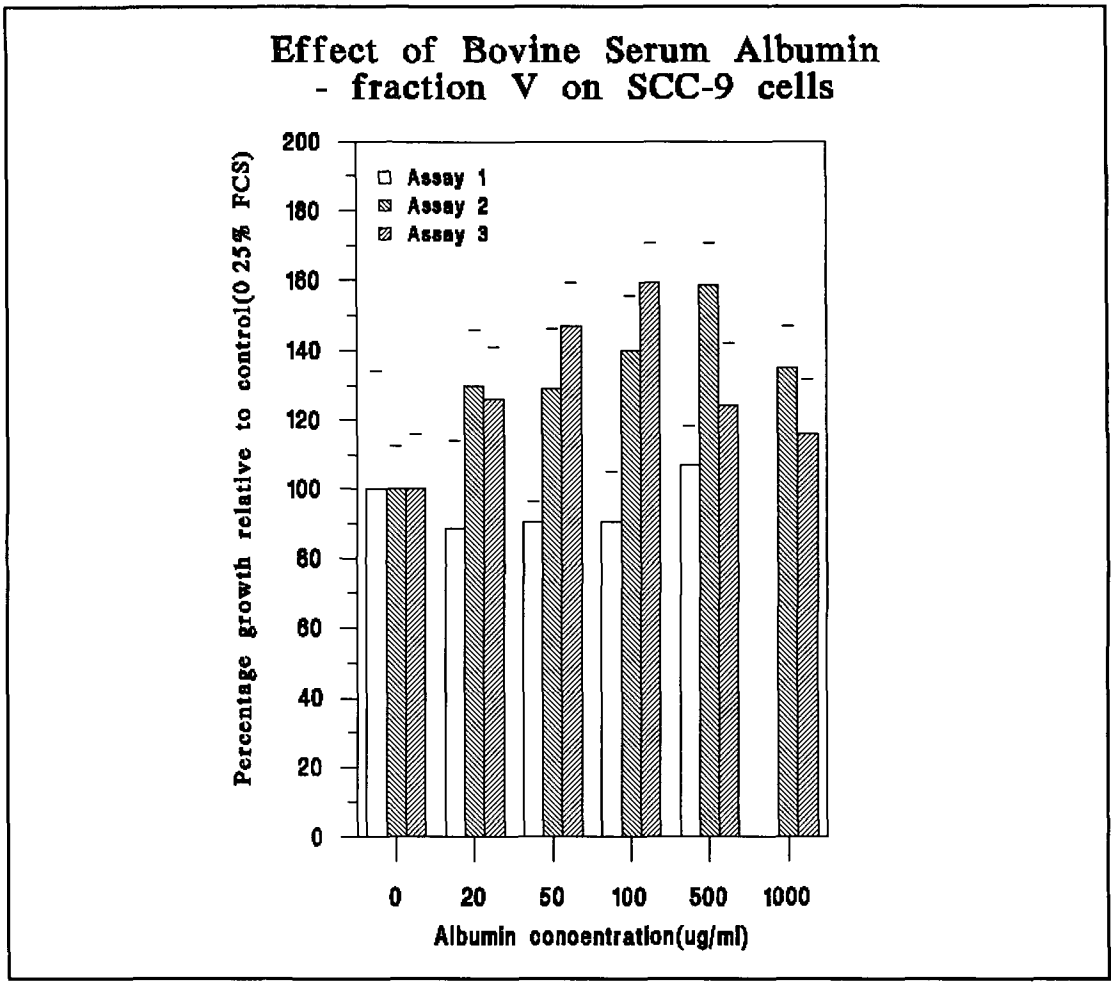


Figure 3.5.1.2.1 shows the growth response of SCC-9 cells to bovine serum albumin - fraction V 1000μg/ml BSA was not tested in Assay 1 Figure and Table 3 5 1 2 1 show results for three independent experiments Results are expressed as the average percentage growth relative to control (0 25% FCS) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point Abbreviations A = albumin, BSA fraction V

Table 3 5 1.2.1 Effect of BSA-fraction V on SCC-9 cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 25% FCS	100 0 $\pm$ 34 4	100 0 $\pm$ 12 4	100 0 $\pm$ 16 0
+ 20μg/ml A	88 69 $\pm$ 25 2	130 0 $\pm$ 16 0	126 2 $\pm$ 14 7
+ 50μg/ml A	90 50 $\pm$ 6 19	128 6 $\pm$ 17 2	147 0 $\pm$ 12 2
+ 100μg/ml A	90 50 $\pm$ 14 4	140 0 $\pm$ 15 6	159 5 $\pm$ 11 3
+ 500μg/ml A	107 0 $\pm$ 11 3	158 6 $\pm$ 11 9	124 0 $\pm$ 18 0
+ 1000μg/ml A	------	135 0 $\pm$ 12 1	116 0 $\pm$ 15 7

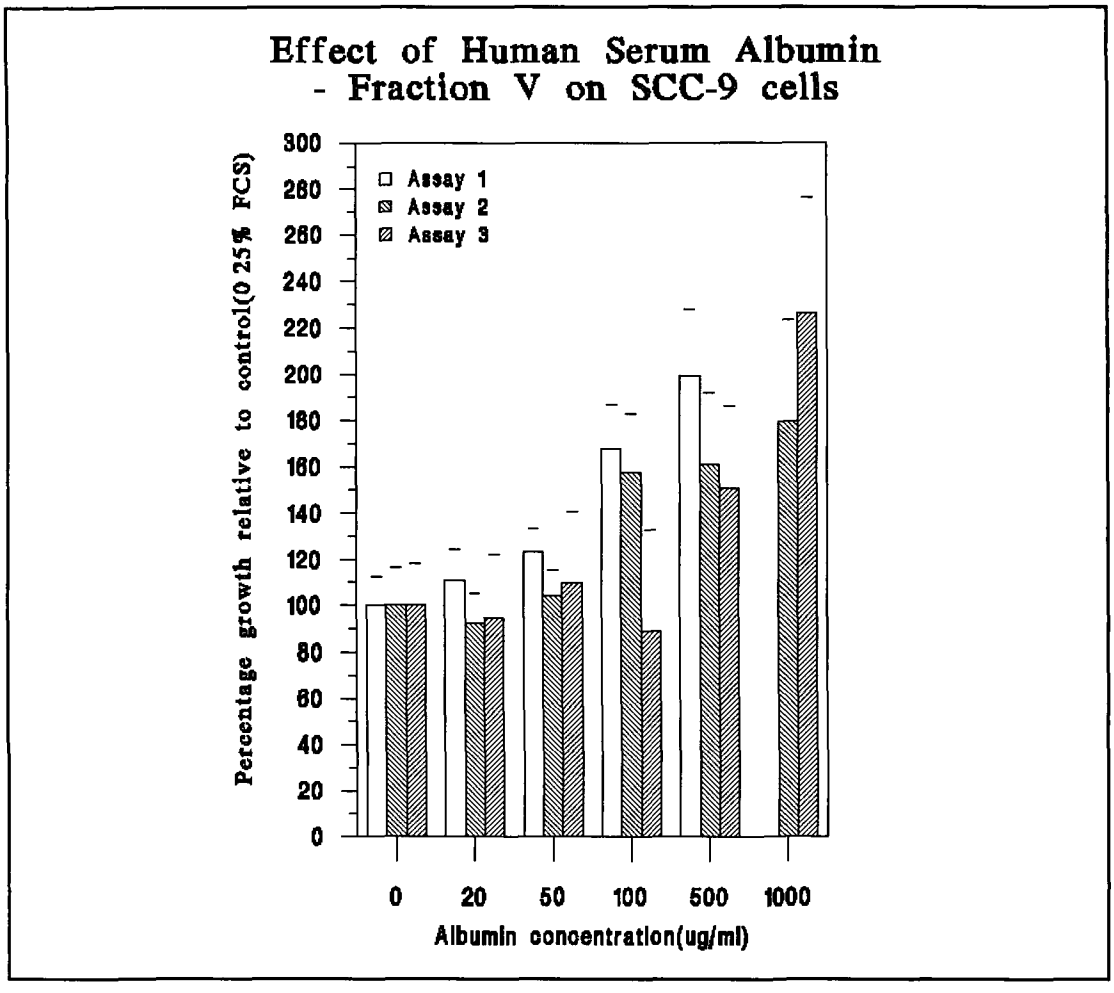


Figure 3 5 1.2.2 shows the growth response of SCC-9 cells to human serum albumin - fraction V 1000μg/ml HSA was not tested in assay 1 Figure and Table 3 5 1 2 2 show the results for three independent experiments Results are expressed as the average percentage growth relative to control (0 25% FCS) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point Abbreviations A = albumin, HSA fraction V

Table 3.5.1.2.2 Effect of HSA-fraction V on SCC-9 cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 25% FCS	100 0 $\pm$ 12 5	100 0 $\pm$ 16 5	100 0 $\pm$ 18 3
+ 20μg/ml A	111 0 $\pm$ 13 1	161 0 $\pm$ 31 1	94 60 $\pm$ 27 6
+ 50μg/ml A	123 0 $\pm$ 10 2	92 10 $\pm$ 13 2	109 9 $\pm$ 30 9
+ 100μg/ml A	168 0 $\pm$ 18 6	104 0 $\pm$ 11 5	88 74 $\pm$ 44 1
+ 500μg/ml A	199 0 $\pm$ 29 1	157 8 $\pm$ 25 3	151 0 $\pm$ 35 3
+ 1000μg/ml A	------	179 6 $\pm$ 43 7	226 2 $\pm$ 50 2

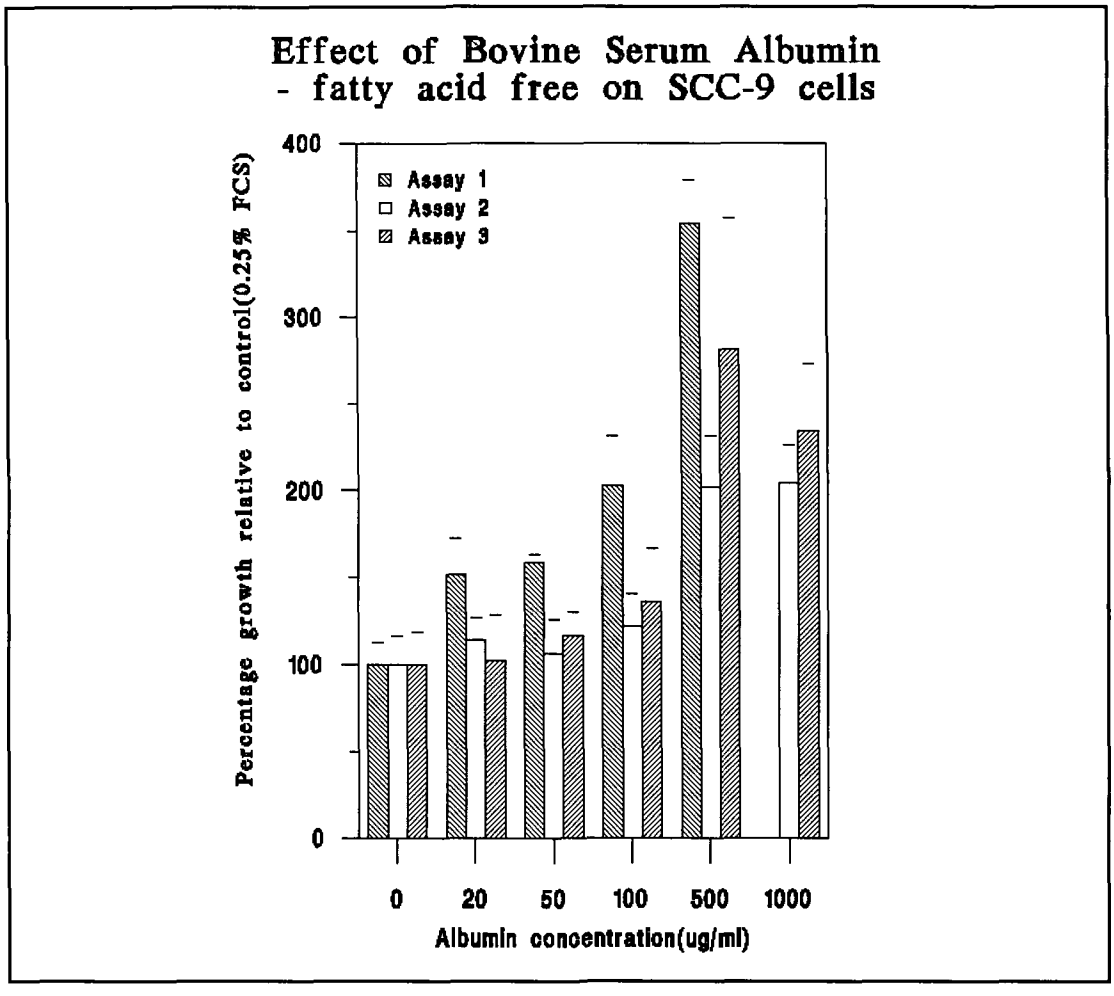


Figure 3.5.1.2.3 shows the growth response of SCC-9 cells to bovine serum albumin - fatty acid free 1000μg/ml BSA was not tested in Assay 1 Figure and Table 3 5 1 2 3 show the results for three independent experiments Results are expressed as the average percentage growth relative control (0 25% FCS) $\pm$ standard deviation (n=8) Acid phosphatase was used as the end point Abbreviations A = albumin, BSA fatty acid free

Table 3.5.1.2.3 Effect of BSA-fatty acid free on SCC-9 cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 25% FCS	100 0 $\pm$ 12 5	100 0 $\pm$ 16 5	100 0 $\pm$ 18 3
+ 20μg/ml A	151 4 $\pm$ 21 1	114 0 $\pm$ 12 6	102 0 $\pm$ 26 5
+ 50μg/ml A	158 7 $\pm$ 4 37	106 0 $\pm$ 19 1	116 7 $\pm$ 12 9
+ 100μg/ml A	202 3 $\pm$ 29 1	121 7 $\pm$ 18 7	135 7 $\pm$ 30 9
+ 500μg/ml A	354 0 $\pm$ 24 4	202 0 $\pm$ 29 1	281 6 $\pm$ 75 5
+ 1000μg/ml A	------	204 0 $\pm$ 22 2	234 0 $\pm$ 38 8

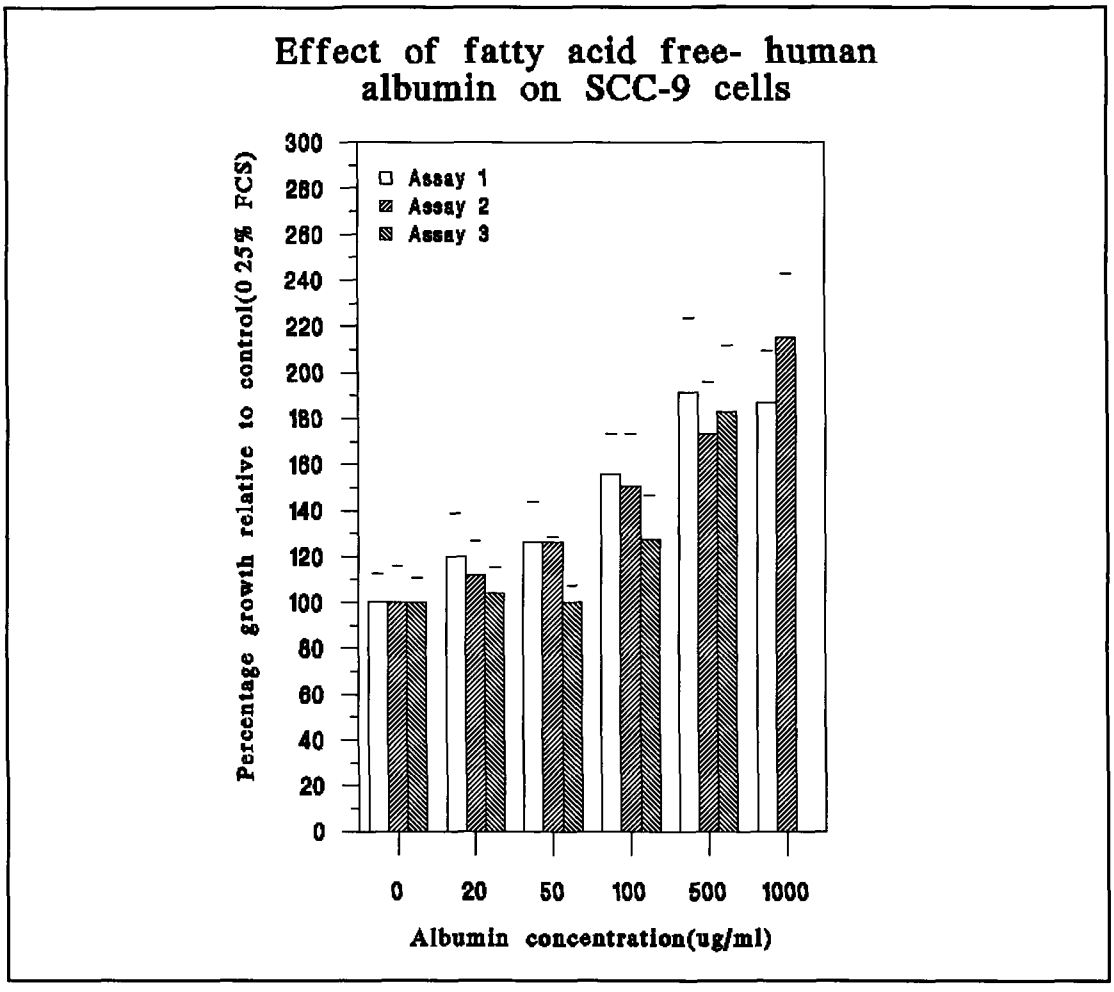


Figure 3.5.1.2.4 shows the growth response of SCC-9 cells to human serum albumin - fatty acid free 1000μg/ml BSA was not tested in Assay 1 Figure and Table 3 5 1 2 4 show results for three independent experiments Results are expressed as the average percentage growth relative to control (0 25% FCS) ± standard deviation (n=8) Acid phosphatase was used as the end point of experiments Abbreviations A = Albumin, HSA fatty acid free

Table 3.5.1.2.4 Effect of HSA-fatty acid free on SCC-9 cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 25% FCS	100 0 ± 10 8	100 0 ± 12 4	100 0 ± 16 0
+ 20μg/ml A	104 0 ± 11 6	119 6 ± 18 6	112 0 ± 14 4
+ 50μg/ml A	100 0 ± 7 30	126 2 ± 17 4	126 0 ± 2 59
+ 100μg/ml A	127 0 ± 19 3	155 7 ± 17 5	150 7 ± 22 2
+ 500μg/ml A	182 5 ± 29 1	191 0 ± 32 8	173 2 ± 22 7
+ 1000μg/ml A	-------	186 4 ± 22 8	214 9 ± 28 2

3.5.1.3 ADDITION OF A LIPID COMPLEX TO HSA-FATTY ACID FREE

As a significant difference in the growth promoting effects was seen between HSA fraction V and HSA fatty acid free for NRK cells, it was decided to investigate the ability of Ex-cyte, a commercially available lipid-protein complex to enhance the activity of HSA fatty acid free A commercial preparation of lipids may partially restore the activity to charcoal treated albumin (Hewlett *et al* , 1989)

The results are shown in Figure 3 5 1 3 and Tables 3 5 1 3a to c The addition of Ex-cyte did not increase the growth response of NRK cells to HSA-faf Some stimulation was seen at the lower concentrations (0 5 - 1 0μg/ml) but no stimulation was seen at higher concentrations The Ex-cyte alone was not stimulatory (Appendix B)

Table 3.5.1.3.1a Effect of combinations of Ex-cyte and HSA fatty acid free on the growth of NRK cells

ASSAY 1	0 1mg/ml A	0 5mg/ml A	1mg/ml A	5mg/ml A
1% DHS	100 0 ± 8 28	100 0 ± 9 58	100 0 ± 9 58	100 0 ± 13 8
0μg/ml Ex*	119 0 ± 13 4	102 7 ± 13 7	73 90 ± 11 5	63 50 ± 10 1
2 5μmg/ml Ex*	128 0 ± 12 4	105 5 ± 8 90	84 90 ± 9 59	73 40 ± 6 14
5μmg/ml Ex*	106 0 ± 11 9	95 20 ± 11 6	83 30 ± 9 88	70 90 ± 8 42
10μg/ml Ex*	105 0 ± 10 9	109 6 ± 10 9	54 80 ± 15 1	75 70 ± 6 13
20μg/ml Ex*	106 0 ± 9 90	96 60 ± 6 85	70 70 ± 7 14	71 20 ± 10 7

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments Abbreviations Ex* = Ex-cyte III, A = Albumin, HSA-faf

Table 3.5.1.3 1b Effect of combinations of Ex-cyte and HSA-fatty acid free on the growth of NRK cells

ASSAY 2	0 1mg/ml A	0 5mg/ml A	1mg/ml A	5mg/ml A
1% DHS	100 0 ± 7 33	100 0 ± 10 4	100 0 ± 10 3	100 0 ± 10 2
0μg/ml Ex*	104 0 ± 11 7	92 10 ± 9 60	68 90 ± 10 3	36 90 ± 3 48
2 5μmg/ml Ex*	103 0 ± 11 3	96 20 ± 7 30	71 80 ± 9 67	45 40 ± 5 08
5μmg/ml Ex*	95 70 ± 11 3	80 50 ± 5 30	57 20 ± 4 36	43 97 ± 5 09
10μg/ml Ex*	94 90 ± 11 7	81 60 ± 8 40	51 70 ± 3 06	44 50 ± 4 02
20μg/ml Ex*	107 0 ± 13 8	80 90 ± 5 99	63 90 ± 9 55	43 70 ± 5 09

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point for experiments Abbreviations Ex* = Ex-cyte III

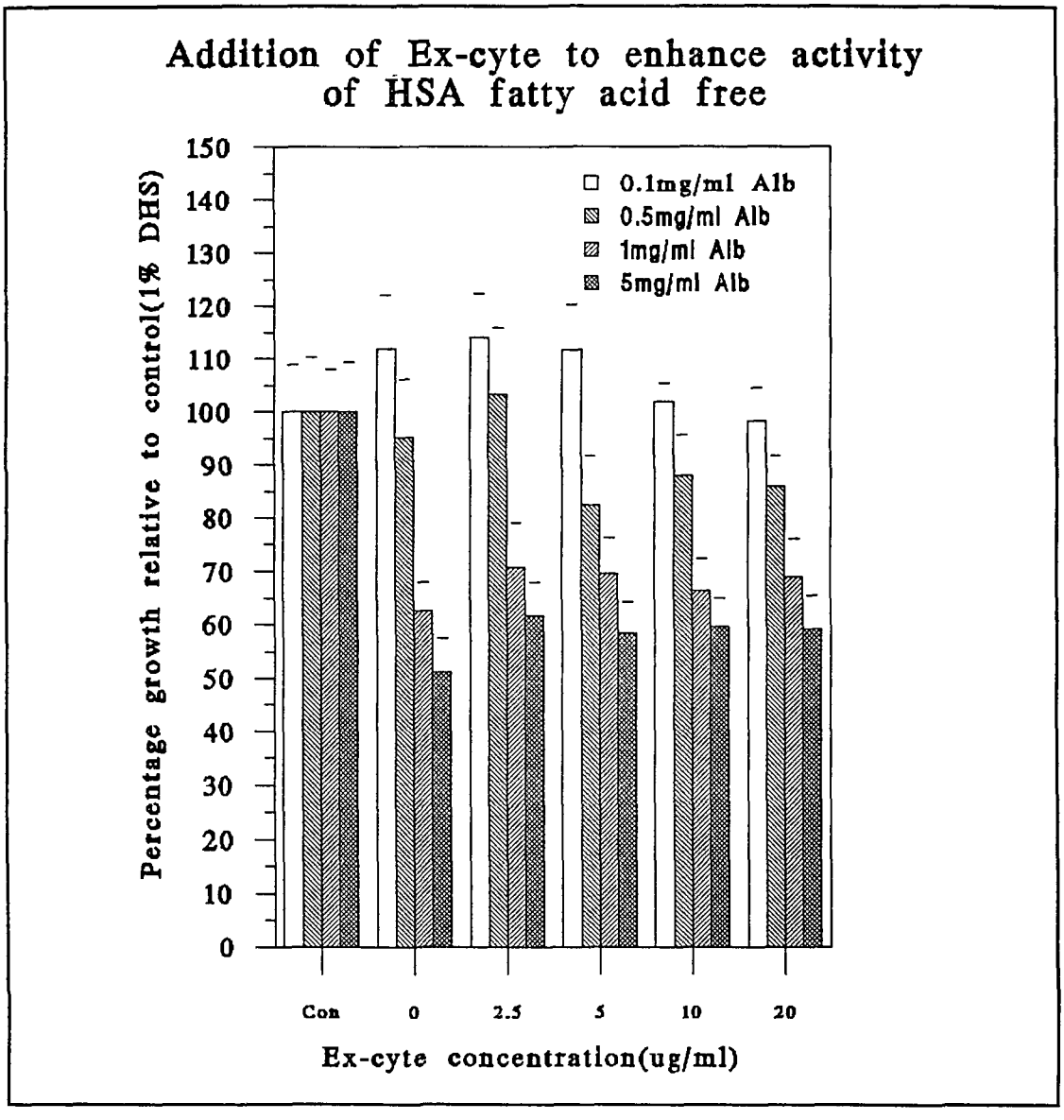


Figure 3.5.1.3.1 shows the effect of the combination of Ex-cyte III and HSA fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) for each experiment Acid phosphatase was used as the end point Tables 3 5 1 3 1a to c show the results for three separate experiments Abbreviations Ex* = Ex-cyte III, A = HSA fatty acid free

Table 3.5.1.3.1c Effect of Ex-cyte on the growth of NRK cells in the presence of HSA-faf

ASSAY 3	0 1mg/ml A	0 5mg/ml A	1mg/ml A	5mg/ml A
1% DHS	100 0 ± 8 90	100 0 ± 10 3	100 0 ± 7 86	100 0 ± 9 30
0μg/ml Ex*	112 0 ± 10 1	95 18 ± 10 9	62 60 ± 5 27	51 20 ± 6 28
2 5μmg/ml	114 0 ± 8 40	103 3 ± 12 6	70 67 ± 8 33	61 75 ± 6 25
5μmg/ml	112 0 ± 8 50	94 20 ± 9 30	69 66 ± 6 74	58 38 ± 5 90
10μg/ml	102 0 ± 3 50	87 85 ± 7 82	66 33 ± 5 94	59 51 ± 5 39
20μg/ml	98 20 ± 6 30	85 80 ± 5 80	68 90 ± 7 24	59 11 ± 6 28

3.5.1.4 LIPID LOADING OF FATTY ACID FREE ALBUMIN

The possibility remained that the presence of fatty acids and /or other lipids could be the source of the activity associated with albumin for the NRK cells even though a commercial lipoprotein complex could not increase the activity of the albumins It was decided therefore, to load some lipid factors directly onto the albumin Oleic acid, cholesterol, phosphatidylserine and phosphatidylcholine were chosen for analysis As some activity was already associated with the bovine-derived fatty acid free albumin, HSA fatty acid free was chosen

Using the Jager method for loading lipids and fatty acids onto albumin, 2 separate runs were carried out Assays 1 and 2 were carried out with complexes from run 1 Assays 3 and 4 were carried out with complexes from run 2 After end over end rotation, the lipid-albumm complex appeared cloudy On filtering through a 0 45μm filter, the solution became clear Fatty acids complexed to albumin are unlikely to be lost during membrane filtration which is routinely used to filter sterilize samples tested in cell culture The cloudiness is believed to be due to uncomplexed lipid droplets/micelles Albumin (*e g* Fraction V) which has a considerable quantitiy of bound lipids, does not form a cloudy solution The loss of turbidity should not have affected the lipid loading drastically To ensure the protein was not lost during filtration, the protein concentrations were measured before and after filtration for the first run and only afterwards for the second run The concentrations are shown in the Tables 3 5 1 4a and b Albumin was gravimetrically determined to be at 1mg/ml

Table 3.5.1.4a Protein concentration of loaded albumin samples used in the first set of experiments

VARIABLES	unfiltered HSA-faf mg/ml	filtered HSA-faf mg/ml
AO1	1 172	1 092
AC1	1 056	1 029
APC0 5	0 979	1 029
APS0 5	1 195	0 947
A	0 851	0 944

Abbreviations AO1 = albumin complexed to Oleic acid (1μg/ml), AC1 = albumin complexed to cholesterol (1μg/ml), APC0 5/APS0 5 = albumin complexed to phosphatidylcholine (0 5μg/ml) or phosphatidylserine(0 5μg/ml) Protein determinations were made using the Biorad method

No significant loss of protein occurred on filtering during run 1 In the second loading experiment, mixtures of the lipids were also loaded onto HSA-faf Table 3 5 1 4b shows the protein profile of the complexes after filtering All albumin solutions were set up so that the final concentration would be 1mg/ml albumin

Table 3.5.1.4b Protein concentration of loaded albumin samples used in second set of experiments

VARIABLES	filtered HSA mg/ml	VARIABLES	filtered HSA mg/ml
C1	0 999	PS1	0 961
C4	0 958	PS5	0 908
C10	1 045	B	0 950
PC0 5	0 945	C	1 101
PC1 0	0 957	D	1 102
PC5	0 972	E	0 969
O1	0 975	F	1 085
O4	1 021	G	1 136
O10	1 071	H	1 029
PS0 5	0 965	I	0 949
		J	0 975

Abbreviations as described in Table 3 5 1 5 1a Protein determinations were made using the Biorad method

Using the samples from the two separate albumin loading runs, two assays per run (4 assays in total) were set up to determine if loading of the lipid factors onto albumin would affect the activity of HSA fatty acid free

3.5.1.4.1 Control plate

Control plates for each of the four experiments were set up to include a serum dilution and the dilution effect of BSA and HSA fraction V Three of the four control plates showed a similar trend (Figure 3 5 1 4 2), while a significantly higher rate of stimulation was seen in the fourth (probably due to a lower initial cell density)

3.5.1.4.2 Cholesterol

Cholesterol alone had no effect on the growth of the cells (Figure 3 5 1 4 2) When allowed to complex overnight to the fatty acid free albumin, some stimulation over the albumin control

subjected to overnight end-over-end rotation was seen The effect was not strong enough to overcome the inhibition of the untreated albumin When cholesterol was added to the albumin without time for overnight complexation, the growth was the same as when the cholesterol was allowed to complex overnight, suggesting no complexation took place or that complexation took place very quickly (*i e* , m both samples, the cholesterol may have been complexed to the albumin) Also, the untreated albumin control which did not undergo overnight rotation at 4°C was less inhibitory than the albumin that did undergo overnight rotation

3.5.1.4.3 Oleic Acid

Oleic acid alone had no stimulatory effect on cell growth (Figure 3 5 1 4 3) When complexed to the fatty acid free albumin, it did not cause any stimulation at 1 and 4μg/ml At 10μg/ml (the highest concentration tested) the combination became inhibitory, while 10μg/ml oleic acid alone was not inhibitory Combination of the oleic acid and albumin just before addition to the cells, showed the same effect as if the combination had been left to complex overnight, indicating that complexation took place very quickly Alternatively, a co-effect may explain the inhibitory effect of the combination of albumin and 10μg/ml oleic acid

3.5.1.4.4 Phosphatidylcholine

Phosphatidylcholine alone did not have any effect on cell growth in the concentration range 0 5 - 5 0μg/ml (Figure 3 5 1 4 5) When allowed to complex to HSA - fatty acid free, there was a slight stimulation, but this was negligible when the standard deviations were taken into account Growth was better when mixing overnight occurred than just combining the two immediatedly before addition to the cells This may have been due to complexation or to some co-effect between phosphatidylcholine and HSA fatty acid free

3.5.1.4.5 Phosphatidylserine

Phosphatidylserine was only assayed twice In these assays, variable results were obtained for the phosphatidylserine alone When complexed to HSA - fatty acid free, stimulation over the control was seen at the lower concentration tested At higher concentrations, the slight stimulatory action was lost

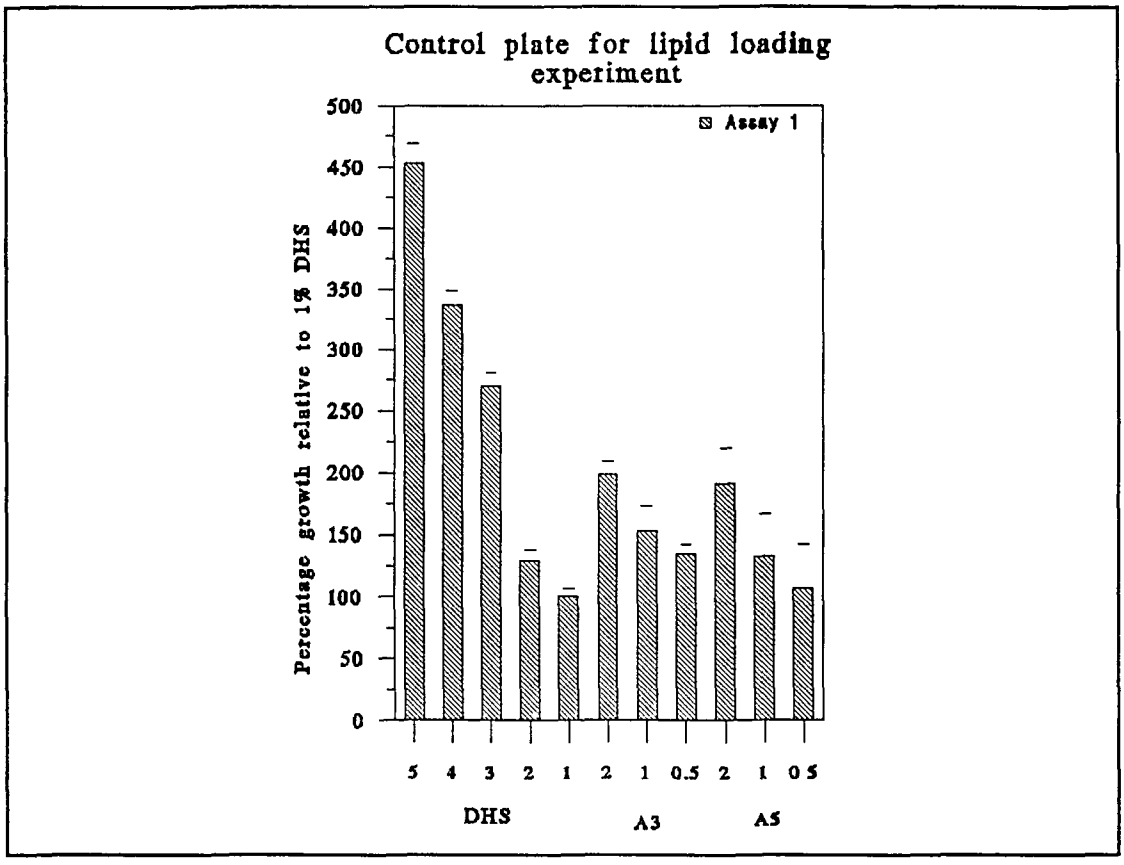


Figure 3.5.1.4.1 shows the control plate from Assay 1 Each assay had a growth control plate, to ensure the cells showed a linear response to serum HSA fraction V as mg/ml A_5 and BSA fraction V as mg/ml A_3 were also included Acid phosphatase was used as the end point Table 3 5 1 4 1 shows the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) for each assay

Table 3.5.1.4.1 Control plate for lipid loading experiments

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
5% DHS	453 0 ± 34 7	387 0 ± 33 7	354 0 ± 27 7	823 0 ± 66 9
4% DHS	337 3 ± 34 4	337 5 ± 31 5	330 5 ± 12 8	628 0 ± 65 7
3% DHS	270 2 ± 28 3	210 0 ± 20 1	153 8 ± 8 16	477 0 ± 23 1
2% DHS	129 5 ± 11 2	161 4 ± 19 6	157 3 ± 8 16	235 0 ± 32 8
1% DHS	100 0 ± 10 1	100 0 ± 10 9	100 0 ± 11 7	100 0 ± 9 33
+2mg/ml A_3	198 0 ± 19 8	402 0 ± 39 4	215 0 ± 17 0	484 0 ± 53 5
+1mg/ml A_3	153 0 ± 8 09	230 0 ± 35 9	177 0 ± 14 4	359 0 ± 42 6
+0 5mg/ml A_3	135 0 ± 15 6	203 0 ± 25 5	164 0 ± 9 56	159 0 ± 37 1
+2mg/ml A_5	191 6 ± 6 65	223 0 ± 21 7	176 0 ± 10 0	236 6 ± 38 3
+1mg/ml A_5	132 4 ± 7 22	207 0 ± 23 4	147 0 ± 18 9	163 5 ± 28 8
+0 5mg/ml A_5	107 0 ± 10 9	188 0 ± 9 56	118 7 ± 8 86	118 0 ± 24 3

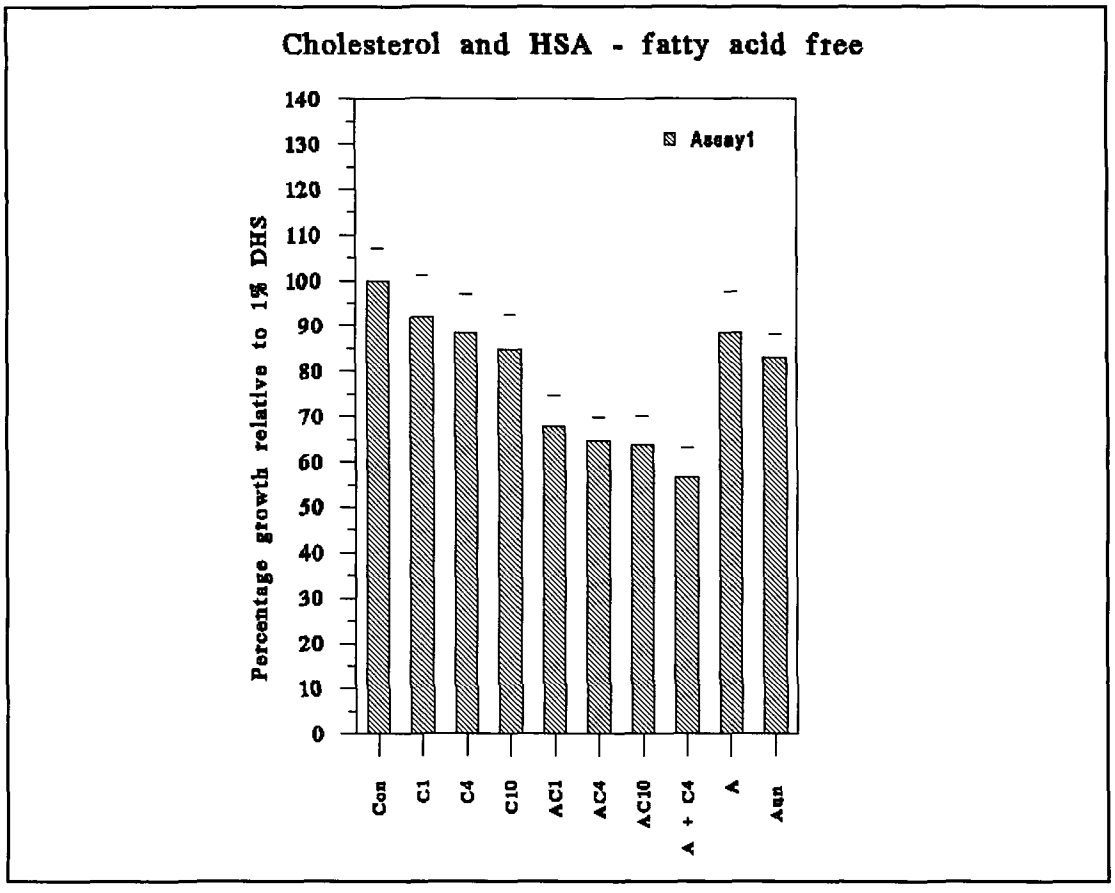


Figure 3.5.1.4.2 shows the effect on growth when cholesterol was complexed to HSA-fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results for each experiment are shown in Table 3 5 1 4 2 Acid phosphatase was used as the end point Assay 2 is not shown Abbreviations C1, C4 and C10 are cholesterol at 1, 4 and 10μg/ml,A = albumin (subjected end-over-end mixing overnight, control for complexation procedure), A+C4 = albumin and cholesterol combined without allowing overnight complexation to occur, Aun = untreated albumin

Table 3.5.1.4.2 Effect of cholesterol complexation to HSA-faf

VARIABLES	ASSAY 1	ASSAY 3	ASSAY 4
1% DHS	100 0 ± 7 24	100 0 ± 7 09	100 0 ± 12 6
+ C1	91 98 ± 9 16	80 93 ± 5 40	109 3 ± 12 6
+ C4	88 54 ± 8 51	102 0 ± 11 2	117 8 ± 12 1
+ C10	84 78 ± 7 69	99 75 ± 10 7	113 5 ± 12 6
+ AC1	67 92 ± 6 71	89 24 ± 8 55	93 46 ± 5 14
+ AC4	64 65 ± 5 24	78 00 ± 5 62	117 3 ± 12 1
+ AC10	63 67 ± 6 55	88 75 ± 6 11	118 7 ± 5 61
+ A	56 48 ± 6 71	71 88 ± 8 80	--------
+ A + C4	88 54 ± 9 16	73 35 ± 6 11	108 8 ± 10 7
+ Aun	83 14 ± 5 07	------	105 1 ± 8 41

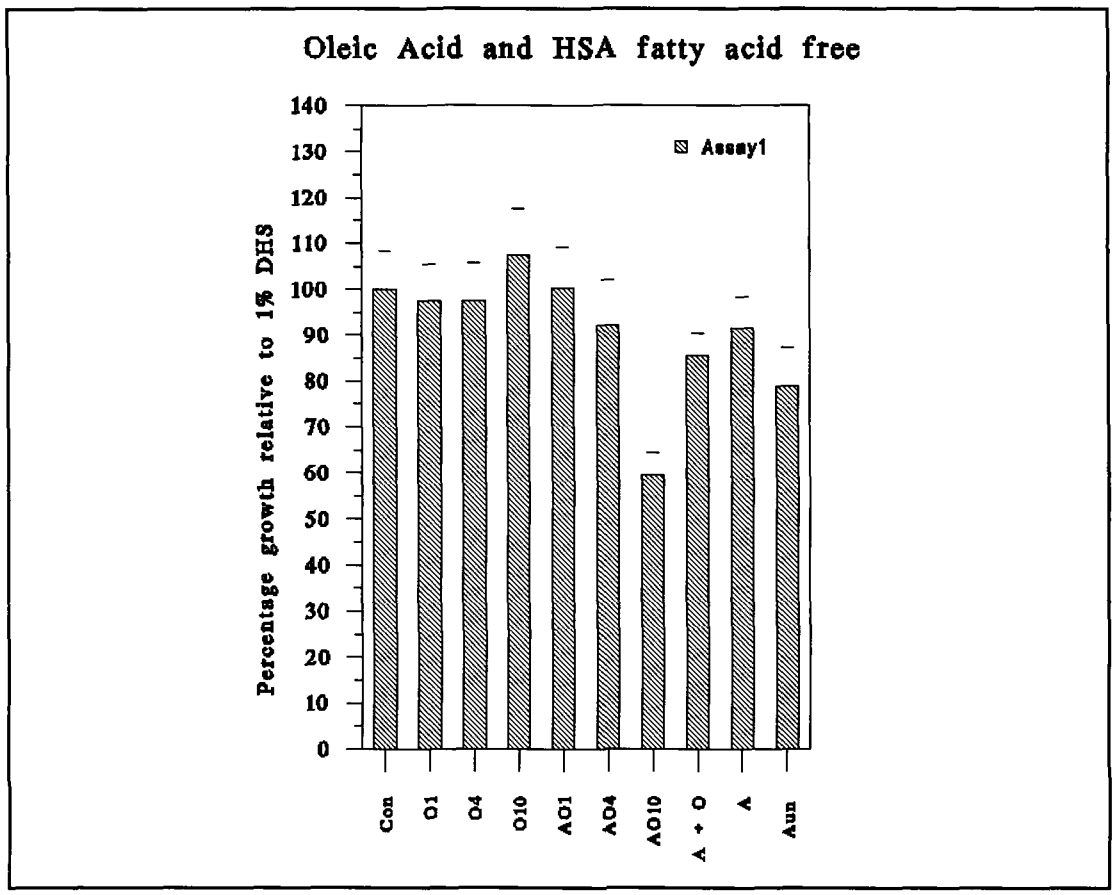


Figure 3 5.1.4.3 shows the effect when oleic Acid was complexed to HSA fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) The results for each assay are shown in Table 3 5 1 4 3 Acid phosphatase was used as the end point Abbreviations O1, O4, and O10 are oleic acid at 1, 4 and 10μg/ml, A = HSA fatty acid free (subjected to end-over-end mixing overnight, control for complexation procedure), A+O4 = albumin and 4μg/ml oleic acid combined without allowing overnight complexation to occur, Aun = untreated HSA fatty acid free

Table 3.5.1.4.3 Effect of oleic complexation to HSA-faf

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
1% DHS	100 0 ± 8 31	100 0 ± 10 3	100 0 ± 7 79	100 0 ± 5 79
+ O1	97 43 ± 7 92	37 94 ± 1 77	92 00 ± 4 93	109 1 ± 12 4
+ O4	97 43 ± 8 14	39 40 ± 8 16	----------	103 0 ± 12 5
+ O10	107 3 ± 10 3	42 55 ± 2 13	87 80 ± 6 34	112 1 ± 14 5
+ AO1	100 4 ± 8 78	31 20 ± 6 74	91 30 ± 7 51	105 5 ± 9 09
+ AO4	92 08 ± 10 1	34 97 ± 6 38	92 00 ± 9 08	126 8 ± 10 4
+ AO10	59 53 ± 4 92	21 63 ± 2 84	58 14 ± 3 03	65 15 ± 11 1
+ A	85 44 ± 4 92	----------	67 60 ± 4 79	----------
+ A + O4	91 43 ± 6 85	91 46 ± 5 67	81 22 ± 7 46	118 2 ± 7 77
+ Aun	78 81 ± 8 56	105 3 ± 10 9	----------	98 99 ± 5 30

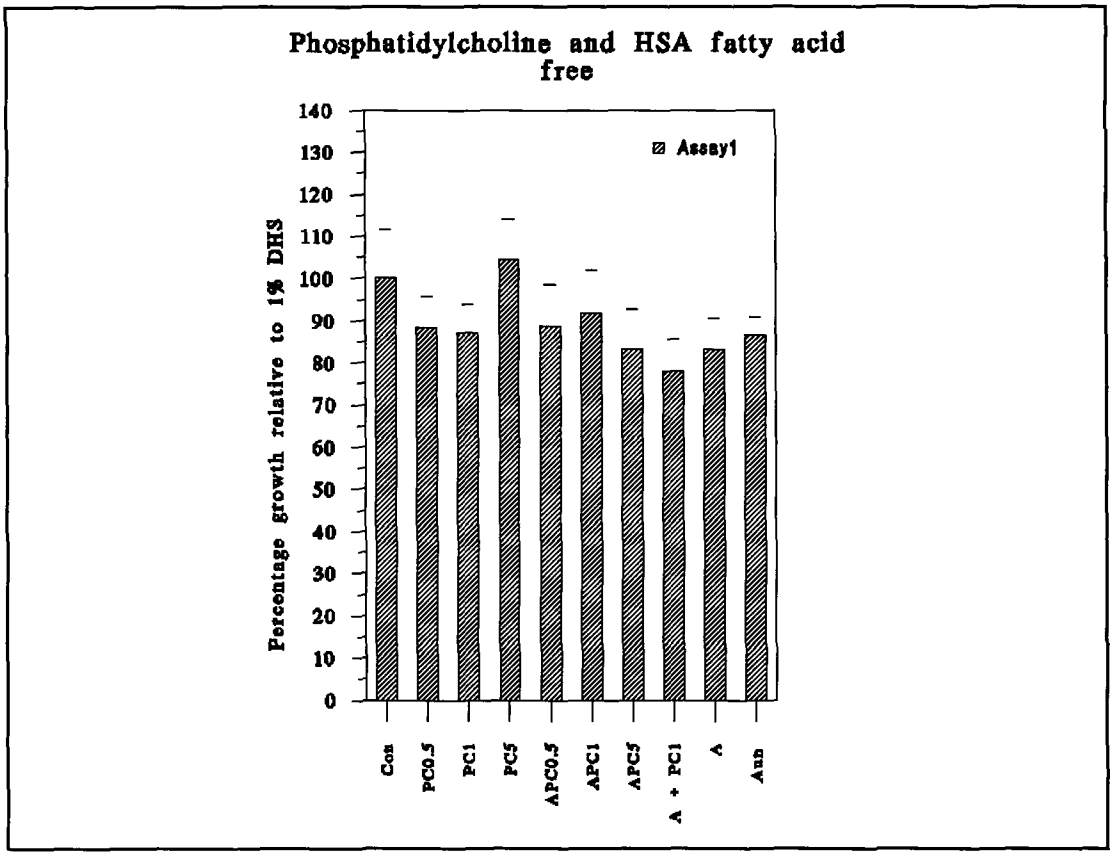


Figure 3.5.1.4.4 shows the effect when phosphatidylcholine was complexed to HSA fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Table 3 5 1 4 4 shows the results for each assay Acid phosphatase was used as the end point Abbreviations PC0 5, PC1, and PC5 are phosphatidylcholine at 0 5, 1 and 5μg/ml, A = HSA fatty acid free (subjected to end-over-end mixing overnight, control for complexation procedure), A+PC1 = albumin and 1μg/ml phosphatidylcholine combined without allowing overnight complexation to occur Aun = untreated HSA fatty acid free

Table 3.5.1.4.4 Effect of phosphatidylcholine complexation to HSA-faf

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
1% DHS	100 0 ± 11 2	100 0 ± 11 3	100 0 ± 4 95	100 0 ± 66 9
+ PC0 5	88 42 ± 7 34	120 0 ± 12 7	92 80 ± 3 01	108 8 ± 65 7
+ PC1	87 06 ± 6 76	110 0 ± 7 60	92 50 ± 10 5	102 7 ± 23 1
+ PC5	104 4 ± 9 65	110 0 ± 8 86	89 70 ± 6 67	101 6 ± 32 8
+ APC0 5	88 80 ± 9 65	140 0 ± 5 06	82 60 ± 3 22	107 7 ± 9 33
+ APC1	91 90 ± 10 2	135 4 ± 11 4	78 30 ± 7 74	102 2 ± 53 5
+ APC0 5	83 20 ± 9 46	133 0 ± 12 7	80 20 ± 5 74	102 7 ± 42 6
+ A	77 99 ± 7 53	----------	65 16 ± 5 16	----------
+ A + PC1	83 20 ± 7 33	120 2 ± 12 7	71 18 ± 4 30	----------
+Aun	80 50 ± 4 44	124 1 ± 16 4	----------	----------

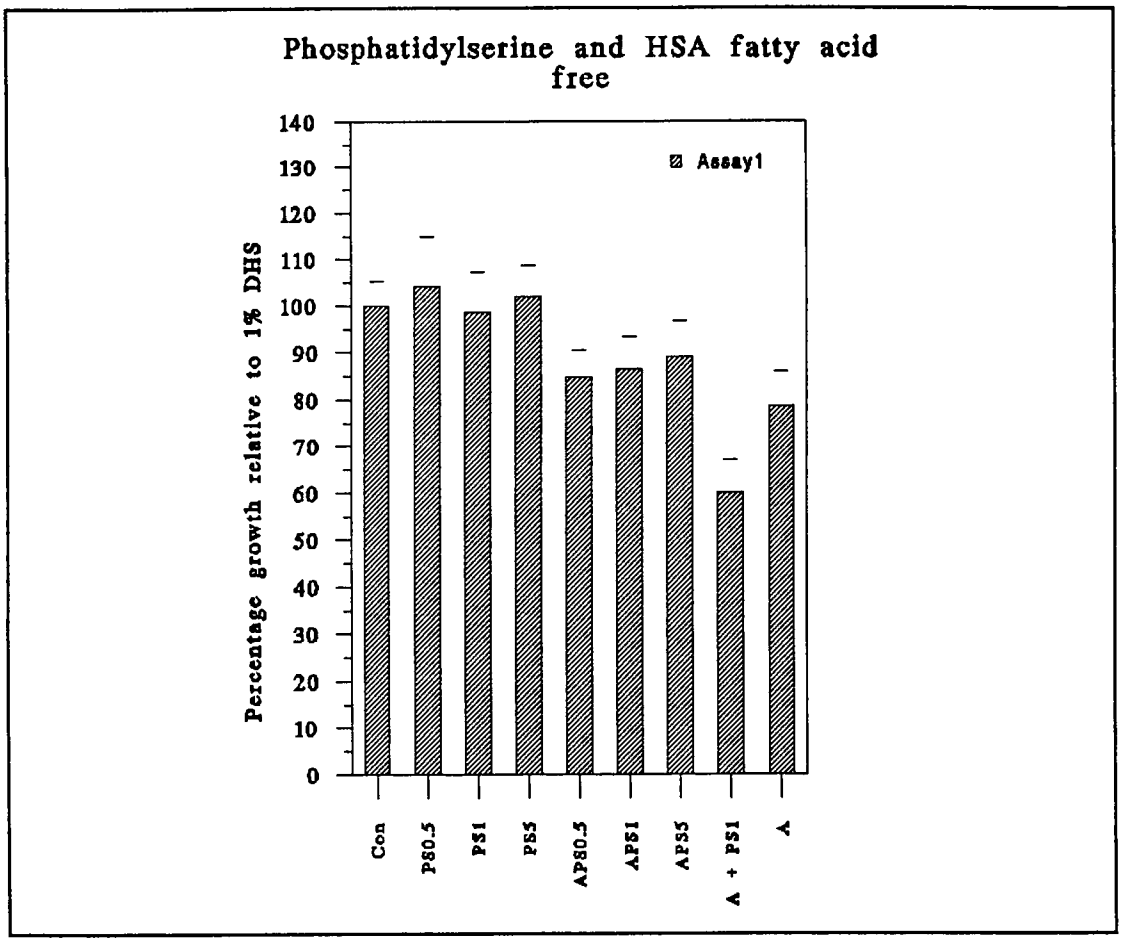


Figure 3.5.1.4.5 shows the effect when phosphatidylserine was complexed to HSA fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) The results for each assay are shown in Table 3 5 1 4 5 Acid phosphatase was used as the end point Abbreviations PS0 5, PS1 and PS5 are phosphatidylserine at 0 5, 1 and 5μg/ml, A = HSA fatty acid free (subjected to end-over-end mixing overnight, control for complexation procedure), A=PS1 = albumin and 1μg/ml phosphatidylserine combined without allowing overnight complexation to occur Aun = untreated HSA fatty acid free

Table 3.5.1.4.5 Effect of phosphatidylserine complexation to HSA-faf

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 5 30	100 0 ± 7 73
+ PS0 5	104 1 ± 10 9	113 0 ± 12 9
+ PS1	98 70 ± 8 57	124 0 ± 4 00
+ PS5	102 0 ± 6 75	127 8 ± 14 6
+ APS0 5	84 93 ± 5 45	139 7 ± 9 60
+ APS1	86 49 ± 6 75	136 0 ± 13 1
+ APS5	89 09 ± 7 53	126 1 ± 14 1
+ A + PS1	68 05 ± 7 01	----------
+ Aun	78 70 ± 7 30	----------

3.5.1.5 COMBINATION OF LIPIDS

The results of combinations of lipids complexed with HSA - fatty acid free and those without are shown in Table 3 5 1 5 1b Table 3 5 1 5 1a shows the composition of the different combinations With or without any albumin, little or no stimulation was seen with any of the factors in combination

From the results obtained in section 3 5 1 4, selected variables were assayed using 24-well plates (Figure 3 5 1 5 2 and 3) The results were read using image analysis and then the crystal violet dye was eluted off the plates and the absorbance was measured on an ELISA plate reader Looking at image analysis as the end point, a combination of both phosphatidylserine and phosphatidylcholine allowed to complex to albumin overnight showed better growth than the combination without overnight mixing The best stimulation of the combinations were with I (4μg/ml oleic acid and 1μg/ml phosphatidylcholine) and J (1μg/ml phosphatidylcholine and 1μg/ml phosphatidylserine) and equal stimulation was effected by phosphatidylserine at 0 5μg/ml - 1μg/ml alone When the dye was eluted off the plate, phosphatidylserine still showed the best stimulation However, the extent of stimulation was lower when dye elution was used as the end point These results would suggest that phosphatidylcholine and phosphatidylserine are more important in cell spreading than in cell division

Table 3.5 1.5 1a Abbreviations of selected variables tested on NRK cells These selected variables were used as the assay conditions in Table 3 5 1 5 1b

VARIABLES	CHOLESTEROL μg/ml	OLEIC ACID μg/ml	PHOSPHATIDYL-CHOLINE μg/ml	PHOSPHATIDYL SERINE μg/ml
B	4	4	-	-
C	4	4	1	-
D	4	4	1	1
E	-	4	1	1
F	-	4	-	1
G	4	-	1	-
H	4	-	-	1
I	-	4	1	-
J	-	-	1	1

Table 3.5.1.5.1b Combinations of lipids with and without HSA fatty acid free

VARIABLES	ASSAY 1		ASSAY 2	
	No albumin	HSA - faf	No albumin	HSA - faf
1% DHS	100 0 ± 12 5	100 0 ± 5 46	100 0 ± 7 32	100 0 ± 8 51
+ B	109 2 ± 10 5	100 0 ± 10 4	98 60 ± 17 4	89 72 ± 7 80
+ C	110 9 ± 10 9	110 0 ± 9 30	95 90 ± 16 7	86 17 ± 9 60
+ D	110 0 ± 17 1	100 0 ± 4 92	106 4 ± 10 7	86 20 ± 8 53
+ E	100 8 ± 13 8	103 3 ± 11 5	113 0 ± 12 4	85 99 ± 3 20
+ F	97 90 ± 7 95	112 2 ± 10 9	107 3 ± 10 8	86 30 ± 6 56
+ G	100 0 ± 7 95	113 7 ± 20 2	106 4 ± 5 26	81 38 ± 4 79
+ H	111 7 ± 9 20	114 2 ± 13 1	113 0 ± 8 47	80 49 ± 6 20
+ I	107 1 ± 12 1	114 7 ± 8 74	111 7 ± 10 1	81 03 ± 6 74
+ J	98 74 ± 8 37	116 4 ± 12 0	99 31 ± 9 70	81 20 ± 3 37

Combinations of lipids were prepared before complexation was allowed to occur Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8 for acid phosphatase)

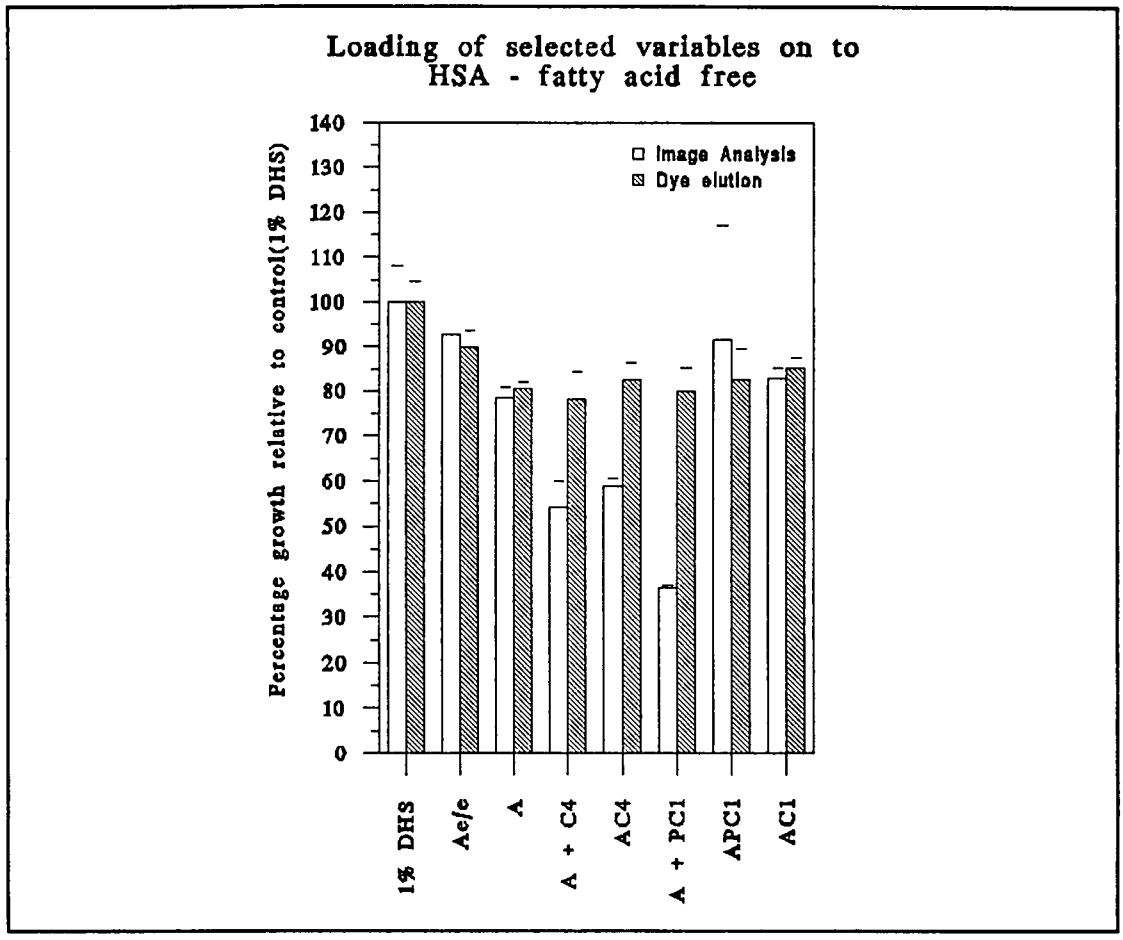


Figure 3.5.1.5.2 shows the response of NRK cells to HSA - fatty acid free loaded with selected variables Results are given as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3 for image analysis and n=6 for dye elution) Results for three independent assays are shown for image analysis (Table 3 5 1 5 2a) and crystal violet dye elution (Table 3 5 1 5 2b) Abbreviations Aun = untreated albumin (no overnight rotation), A = albumin subjected to end-over-end mixing overnight, control for complexation procedure All other abbreviations are as described in Tables 3 5 1 4 2 and 3 5 1 4 4

Table 3.5.1.5.2a Effect of HSA-faf complexed to selected variables as determined by image analysis

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 7 95	100 0 ± 25 0	100 0 ± 10 9
+ Aun	92 70 ± 0 06	77 93 ± 4 87	206 3 ± 7 54
+ A	78 60 ± 2 34	87 11 ± 10 3	154 6 ± 12 1
+ A + C4	54 30 ± 5 75	63 47 ± 14 3	192 3 ± 21 9
+ AC4	58 80 ± 1 72	65 57 ± 13 2	205 6 ± 8 89
+ A + PC1	36 50 ± 0 71	88 76 ± 25 4	108 0 ± 28 2
+ APC1	91 65 ± 25 3	193 0 ± 46 2	215 9 ± 23 0
+ AC1	82 85 ± 2 30	152 7 ± 28 9	240 7 ± 0 33

Table 3.5.1.5.2b Effect of HSA-faf complexed to selected variables as determined by dye elution

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 4 67	100 0 ± 2 14	100 0 ± 3 55
+ Aun	89 92 ± 3 77	97 11 ± 9 61	110 4 ± 12 6
+ A	80 56 ± 1 48	97 25 ± 4 26	94 35 ± 8 58
+ A + C4	78 34 ± 5 95	96 01 ± 2 78	102 2 ± 7 81
+ AC4	82 43 ± 3 83	109 3 ± 2 13	118 6 ± 4 57
+ A + PC1	79 92 ± 5 27	108 3 ± 9 12	92 39 ± 4 97
+ APC1	82 64 ± 6 77	117 1 ± 10 5	112 5 ± 0 02
+ AC1	85 21 ± 2 22	107 8 ± 8 99	117 7 ± 7 42

Table 3.5.1.5.3a Effect of HSA-faf complexed to selected variables as determined by Image analysis

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 8 92	100 0 ± 5 36	100 0 ± 5 79
+ Aun	84 40 ± 8 50	79 40 ± 19 4	212 3 ± 3 15
+ A	103 3 ± 0 06	43 11 ± 3 69	192 1 ± 1 31
+ AI	142 4 ± 10 6	5 350 ± 0 16	146 3 ± 55 6
+ AJ	152 0 ± 4 98	63 24 ± 12 4	148 2 ± 5 88
+ APS0 5	132 0 ± 10 7	65 29 ± 2 56	221 8 ± 34 6
+ APS1	101 0 ± 17 8	50 50 ± 1 00	250 2 ± 11 2
+ A + PS1	95 00 ± 18 7	40 57 ± 8 63	228 9 ± 25 7

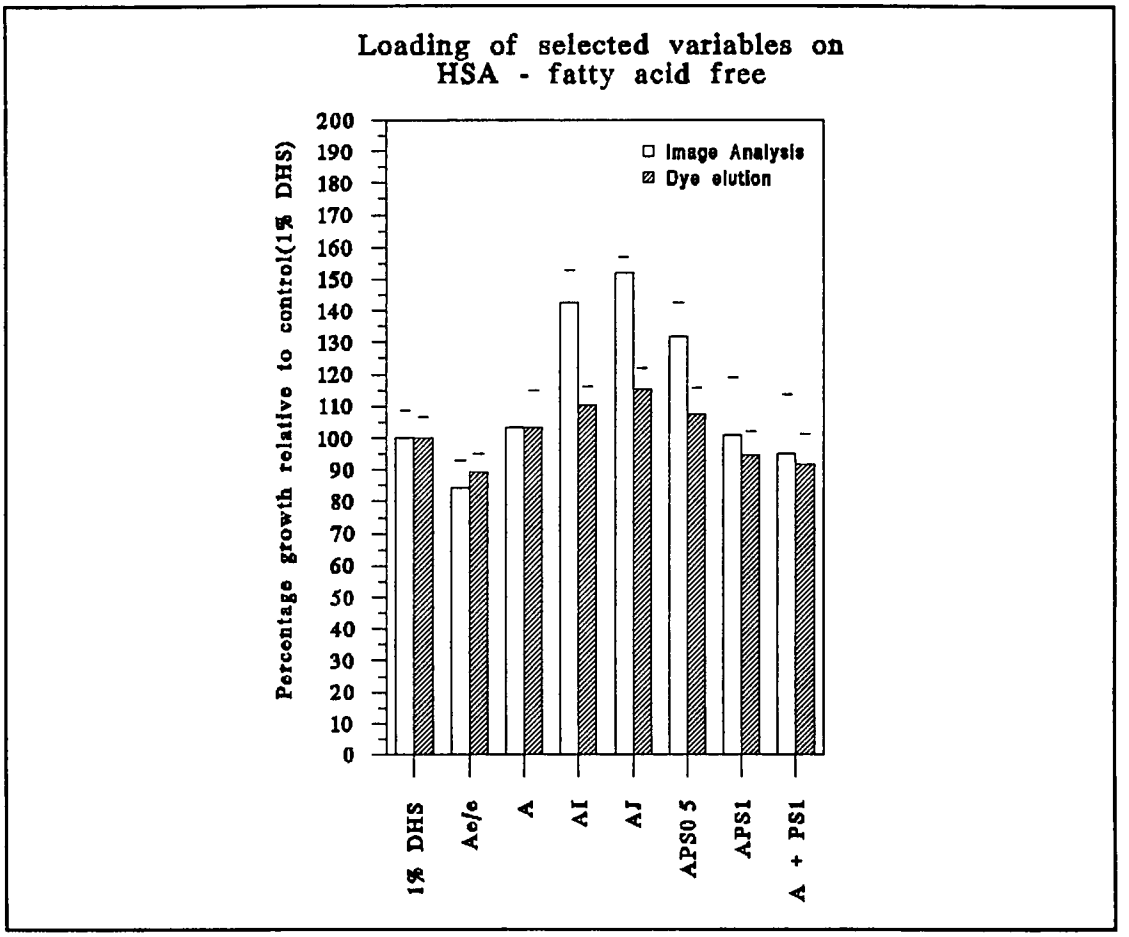


Figure 3.5.1.5.3 shows the response of NRK cells to HSA - fatty acid free loaded with selected variables Results are given as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3 for image analysis and n=6 for dye elution) Results for three independent assays are shown for image analysis (Table 3 5 1 5 3a) and crystal violet dye elution (Table 3 5 1 5 3b) Abbreviations Aun = untreated albumin (no overnight rotation), A = albumin subjected to end-over-end mixing overnight, control for complexation procedure All other abbreviations are as described in Tables 3 5 1 4 5 and 3 5 1 5 1a

Table 3.5.1.5.3b Effect of HSA-faf complexed to selected variables as determined by dye elution

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 6 51	100 0 ± 4 30	100 0 ± 9 16
+ Aun	89 14 ± 5 72	88 88 ± 0 84	98 67 ± 5 65
+ A	103 4 ± 11 4	83 74 ± 4 20	109 4 ± 6 58
+ AI	110 4 ± 5 91	72 19 ± 4 29	94 46 ± 7 43
+ AJ	115 5 ± 6 44	92 33 ± 4 08	107 3 ± 5 21
+ APS0 5	107 4 ± 8 28	95 39 ± 1 66	121 1 ± 4 00
+ APS1	94 50 ± 7 64	90 97 ± 3 59	112 5 ± 12 5
+ A + PS1	91 72 ± 9 40	71 73 ± 8 46	109 5 ± 5 70

3.5.2 EFFECT OF DIFFERENT BATCHES OF ALBUMINS

The results obtained in section 3.5.1, showed variations in the growth stimulatory ability of fraction V and fatty acid free albumins (both BSA and HSA). That fatty acid free albumins were less stimulatory for NRK cells than fraction V suggested that fatty acids or some other lipid removed during charcoal treatment may be responsible for the loss of activity. Combination of HSA fatty acid free with specific lipids did not restore activity.

In order to find out if differences in the batches of albumin used in section 3.5.1 were responsible for the differences in the observed activity between the fraction V and the fatty acid free albumins, three albumins and the fatty acid free albumins derived from these 3 albumins were tested on NRK cells. Fraction Vs with commercially available fatty acid free albumins were tested; hence the albumins used here were not the same as those in section 3.5.1 (fatty acid free derived albumins were not available). In addition, equine serum albumin (ESA) and its fatty acid free derivative were tested because the background serum was donor horse. The assays were set up in 96-well plates. In the first of three separate experiments, duplicate plates were set up. One of each of the plates were taken down with acid phosphatase and the second with dye elution. The results are shown in Figures 3.5.2.1 to 6.

3.5.2.1 BSA fraction V and fatty acid free

The growth response of BSA (Sigma A4503, lot 12H0283) is shown in Figure 3.5.2.1.1. The fraction V shows growth stimulation with both AP and DE as end points, but the extent of stimulation obtained with AP was much less than that obtained with DE. The fatty acid free albumin (Sigma A6003, lot 119F9306) derived from this fraction V was inhibitory (as had been observed with HSA-faf as described in section 3.5.1.1.3). Increasing the albumin concentration resulted in increased inhibition with a maximum inhibition of 23 - 24% of control (1% DHS) at 5mg/ml (Figure 3.5.2.1.2).

3.5.2.2 HSA fraction V and fatty acid free

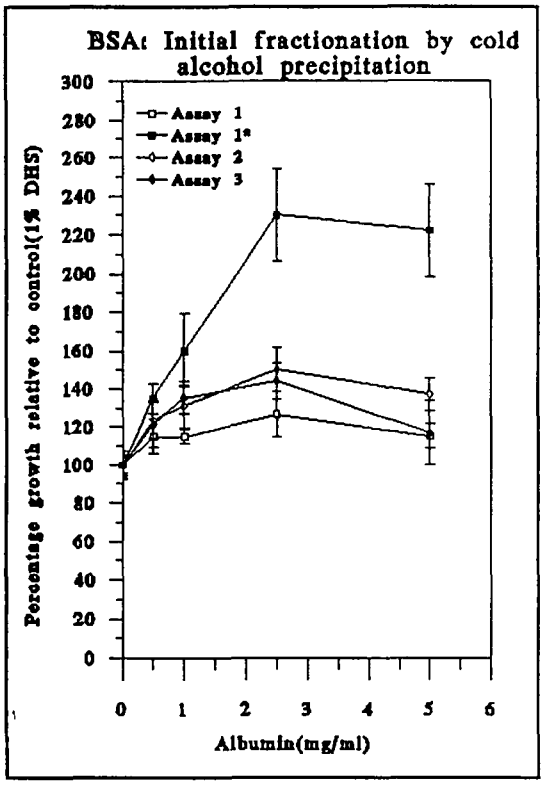
For the fraction V albumin (Sigma A1653, lot 106F9333), good stimulation was seen with both end points (Figure 3.5.2.2.1). Dye elution showed only slightly better stimulation than acid phosphatase. Stimulation increased with increasing concentration to reach a maximum of 2.3-fold to 3.1-fold stimulation over the control (1% DHS) at 5mg/ml. The fatty acid free albumin

(Sigma A1887, lot 42H9313) derived from this fraction V showed little or no effect in two of the three assays (Figure 3 5 2 2 2) For the duplicate plate from assay 1 that was taken down with dye elution, a slight stimulation was seen while no effect was seen with AP as the end point

3.5.2.3 ESA fraction V and fatty acid free

For fraction V (Sigma A9888, lot 37F9326), very little stimulation was seen Stimulation reached a maximum of 30% relative to the control (1% DHS) at 1 0mg/ml while at 5mg/ml, inhibition was seen (Figure 3 5 2 3 1) For the fatty acid free albumin (Sigma A5280, lot 47F9303) derived from this fraction V, slight stimulation of 15 - 40% above the control was seen depending on the assay at 2 5 - 5 0mg/ml (Figure 3 5 2 3 2) Unlike the fraction V, it was not inhibitory but there was a fall in stimulation at the highest concentration tested (5mg/ml)

From these results, it was seen that the charcoal treatment resulted in a loss of activity for the BSA and HSA tested This loss in activity may have been due to the removal of fatty acids/lipids or to the charcoal treatment destroying the activity of some other factor For equine serum albumin, the little activity was seen in the fraction V was not lost on charcoal treatment, however the inhibitory effect at higher concentrations was lost This may have been due to the removal of some lipids or fatty acids that were inhibitory to NRK cells This indicated that for some batches of albumin the presence of fatty acids might be important for growth while for other batches, some other variable appeared to be affecting the cells



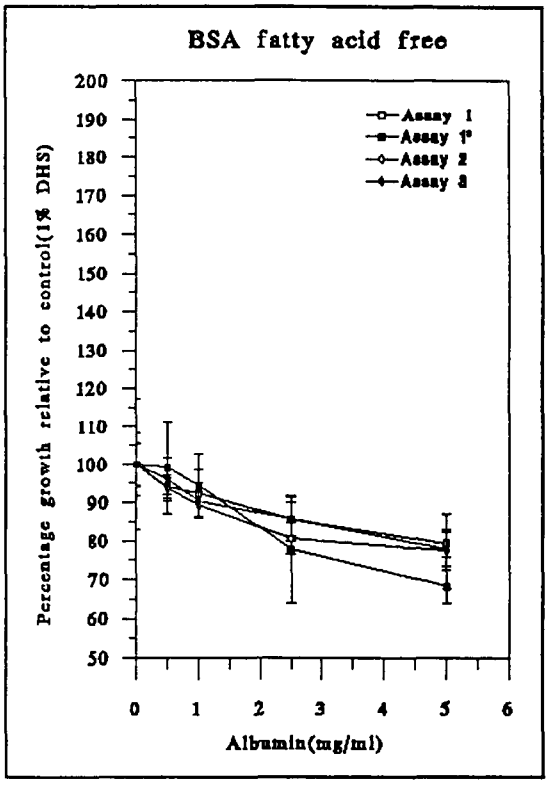


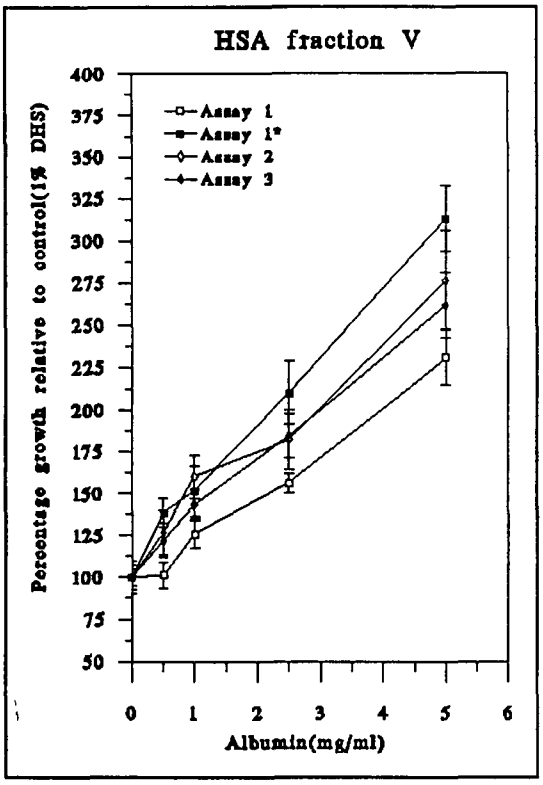
Figure 3.5.2.1.1 and 3.5.2 1.2 show the growth response of NRK cells to BSA fraction V and fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point, except for Assay 1* This assay was from the same experiment as Assay 1 but was taken down with dye elution as the end point Tables 3 5 2 1 1 and 3 5 2 1 2 show the results for the different fractions Abbreviation A = Albumin

Table 3.5.2.1.1 Growth response of NRK cells to BSA fraction V (Sigma 4503)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 7 49	100 0 ± 7 09	100 0 ± 5 44	100 0 ± 4 13
+ 0 5mg/ml A	115 1 ± 9 43	134 9 ± 7 93	123 4 ± 9 54	121 4 ± 12 2
+ 1 0mg/ml A	115 1 ± 3 77	160 0 ± 19 1	131 1 ± 11 1	135 4 ± 8 35
+ 2 5mg/ml A	126 6 ± 11 7	230 2 ± 23 8	150 3 ± 11 6	144 2 ± 9 48
+ 5 0mg/ml A	115 1 ± 6 60	222 2 ± 23 8	137 1 ± 8 50	116 7 ± 17 1

Table 3.5.2.1 2 Growth response of NRK cells to BSA fatty acid free (Sigma, A6003)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 5 86	100 0 ± 17 1	100 0 ± 8 25	100 0 ± 5 53
+ 0 5mg/ml A	94 30 ± 2 33	99 19 ± 12 1	93 72 ± 3 40	96 39 ± 5 26
+ 1 0mg/ml A	92 33 ± 6 33	94 35 ± 8 14	89 27 ± 3 40	90 58 ± 4 71
+ 2 5mg/ml A	85 67 ± 5 67	77 82 ± 14 1	80 63 ± 4 19	85 87 ± 4 43
+ 5 0mg/ml A	79 33 ± 3 67	68 22 ± 4 33	77 49 ± 9 68	77 84 ± 4 43



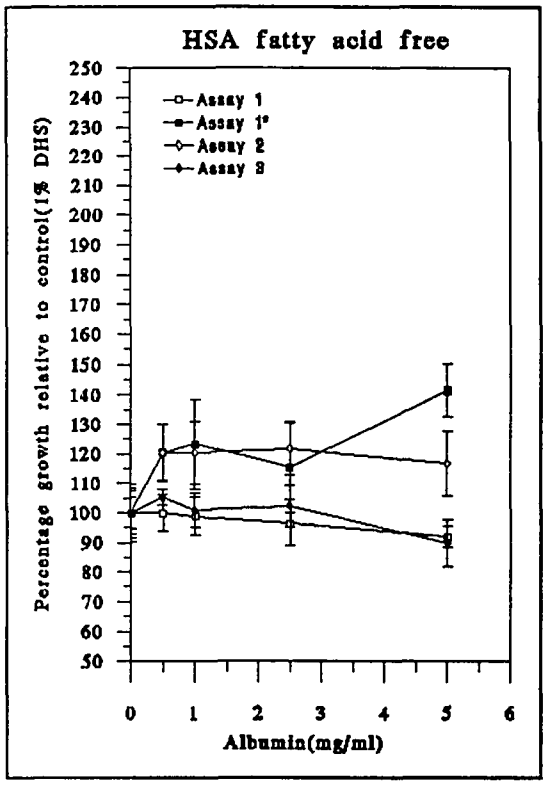


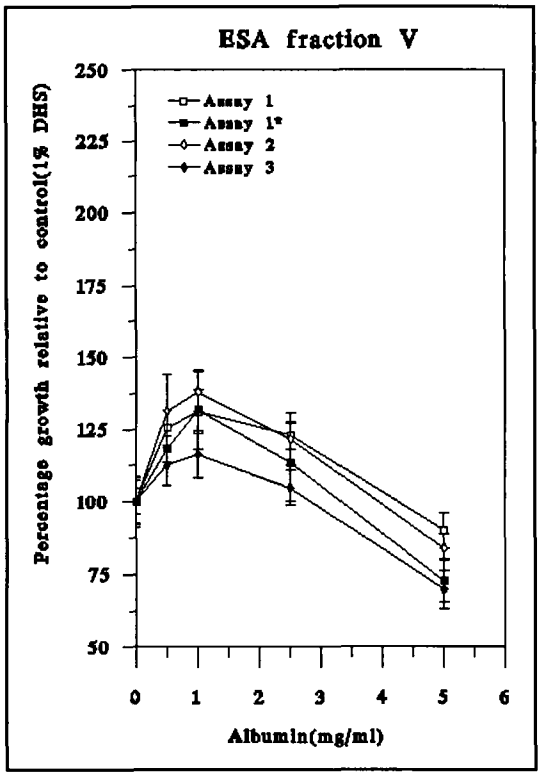
Figure 3.5.2.2.1 and 3 5.2.2.2 show the growth response of NRK cells to the HSA fraction V and fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point, except for Assay 1* This assay was from the same experiment as Assay 1 but was taken down with dye elution as the end point Tables 3 5 2 2 1 and 3 5 2 2 2 show the results for the different fractions Abbreviation A = mg/ml albumin

Table 3.5.2.2.1 Growth response of NRK cells to HSA fraction V (Sigma A1653)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 5 28	100 0 ± 9 73	100 0 ± 7 35	100 0 ± 5 41
+ 0 5mg/ml A	101 2 ± 7 69	138 5 ± 8 46	126 8 ± 13 4	122 0 ± 10 0
+ 1 0mg/ml A	125 9 ± 8 61	151 6 ± 15 2	160 0 ± 13 0	143 4 ± 9 21
+ 2 5mg/ml A	156 2 ± 5 89	209 9 ± 18 7	182 5 ± 17 9	184 5 ± 13 3
+ 5 0mg/ml A	230 9 ± 16 3	312 8 ± 19 3	276 3 ± 29 7	261 5 ± 19 5

Table 3.5.2.2.2 Growth response of NRK cells to HSA fatty acid free (Sigma A1887)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 5 28	100 0 ± 9 73	100 0 ± 7 35	100 0 ± 5 41
+ 0 5mg/ml A	100 0 ± 6 34	120 5 ± 9 32	120 3 ± 9 89	105 1 ± 2 71
+ 1 0mg/ml A	98 71 ± 6 34	123 1 ± 15 4	120 3 ± 10 5	100 8 ± 5 69
+ 2 5mg/ml A	96 45 ± 7 69	115 4 ± 15 4	121 6 ± 8 90	102 2 ± 7 05
+ 5 0mg/ml A	91 92 ± 3 62	141 5 ± 8 77	116.7 ± 11.2	89 90 ± 7 86



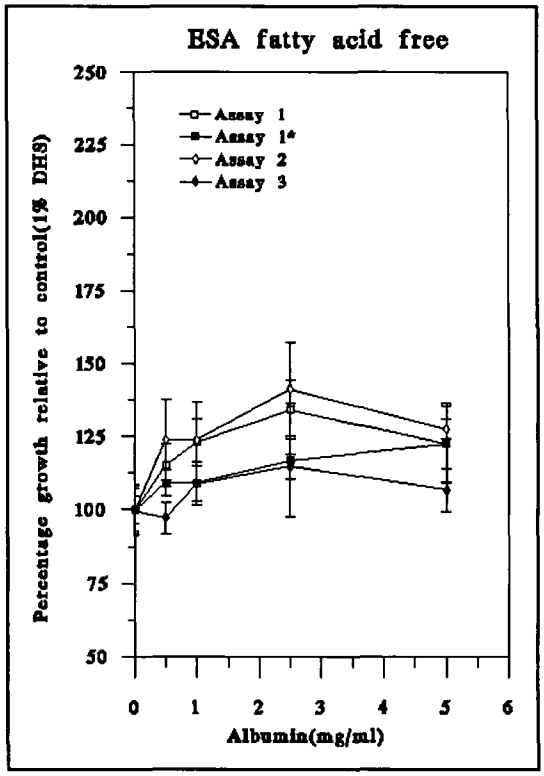


Figure 3.5.2.3.1 and 3.5.2.3.2 show the growth response of NRK cells to ESA fraction V and fatty acid free Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point, except for Assay 1* This assay was from the same experiment as Assay 1 but was taken down with dye elution as the end point Tables 3 5 2 3 1 and 3 5 2 3 2 show the results for the different fractions Abbreviation A = equine serum albumin

Table 3 5.2.3.1 Growth response of NRK cells to ESA fraction V (Sigma A9888)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 8 79	100 0 ± 7 36	100 0 ± 8 05	100 0 ± 4 49
+ 0 5mg/ml A	125 5 ± 6 06	118 2 ± 4 54	131 3 ± 12 8	112 5 ± 7 09
+ 1 0mg/ml A	131 2 ± 7 79	131 8 ± 13 6	138 1 ± 7 02	116 3 ± 8 27
+ 2 5mg/ml A	112 9 ± 4 76	113 6 ± 13 6	121 5 ± 9 02	104 7 ± 6 15
+ 5 0mg/ml A	90 04 ± 6 06	72 73 ± 7 36	84 16 ± 4 49	69 74 ± 6 38

Table 3.5.2.2.3.2 Growth response of NRK cells to ESA fatty acid free (Sigma A5280)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 8 79	100 0 ± 7 36	100 0 ± 8 05	100 0 ± 4 49
+ 0 5mg/ml A	115 1 ± 7 36	109 1 ± 4 54	123 8 ± 14 0	97 16 ± 5 44
+ 1 0mg/ml A	123 1 ± 13 6	109 1 ± 7 36	123 7 ± 7 44	108 9 ± 6 15
+ 2 5mg/ml A	134 2 ± 9 96	116 9 ± 19 4	141 3 ± 16 0	114 8 ± 4 29
+ 5 0mg/ml A	122 6 ± 12 9	122 7 ± 13 6	127 6 ± 3 49	106 8 ± 7 33

3.5.2.4 EFFECT OF INITIAL ISOLATION STEP ON ALBUMIN ACTIVITY

To see if the method of isolation could be related to variations in activity, albumin prepared using the three most commonly used initial isolation steps, cold alcohol precipitation, heat shock and salt fractionation, were tested The results are shown in Figures 3 5 2 4 1 to 3

As seen in section 3 5 2 4 1 cold alcohol precipitation was the method of initial fractionation used for BSA fraction V in the previous two sections Marginal stimulation of 26 - 50% relative to the control was observed with acid phosphatase (Figure 3 5 2 4 1) For dye elution, the extent of stimulation was much higher, reaching a maximum of 2 3-fold stimulation over the control (1% DHS) at 2 5mg/ml The results for dye elution corresponded favourably with the results obtained in section 3 5 1 1 and 3 5 1 2 1 When salt fractionation was used as the initial fractionation step (Figure 3 5 2 4 2), a total loss in stimulation was observed, however no inhibition was seen For heat-shock treatment the stimulation was much higher than that seen previously (Figure 3 5 2 4 3) Increasing the concentration resulted in increased growth reaching a maximum of 3 5-fold to 4 3-fold stimulation over the control at 5 0mg/ml (the highest concentration tested) This is higher than the activity seen with BSA in the section 3 5 1

These results show that the activity observed can depend on the initial fractionation step However, only one batch from each initial fractionation step was tested, so there was no way of knowing if they were from the same batch originally

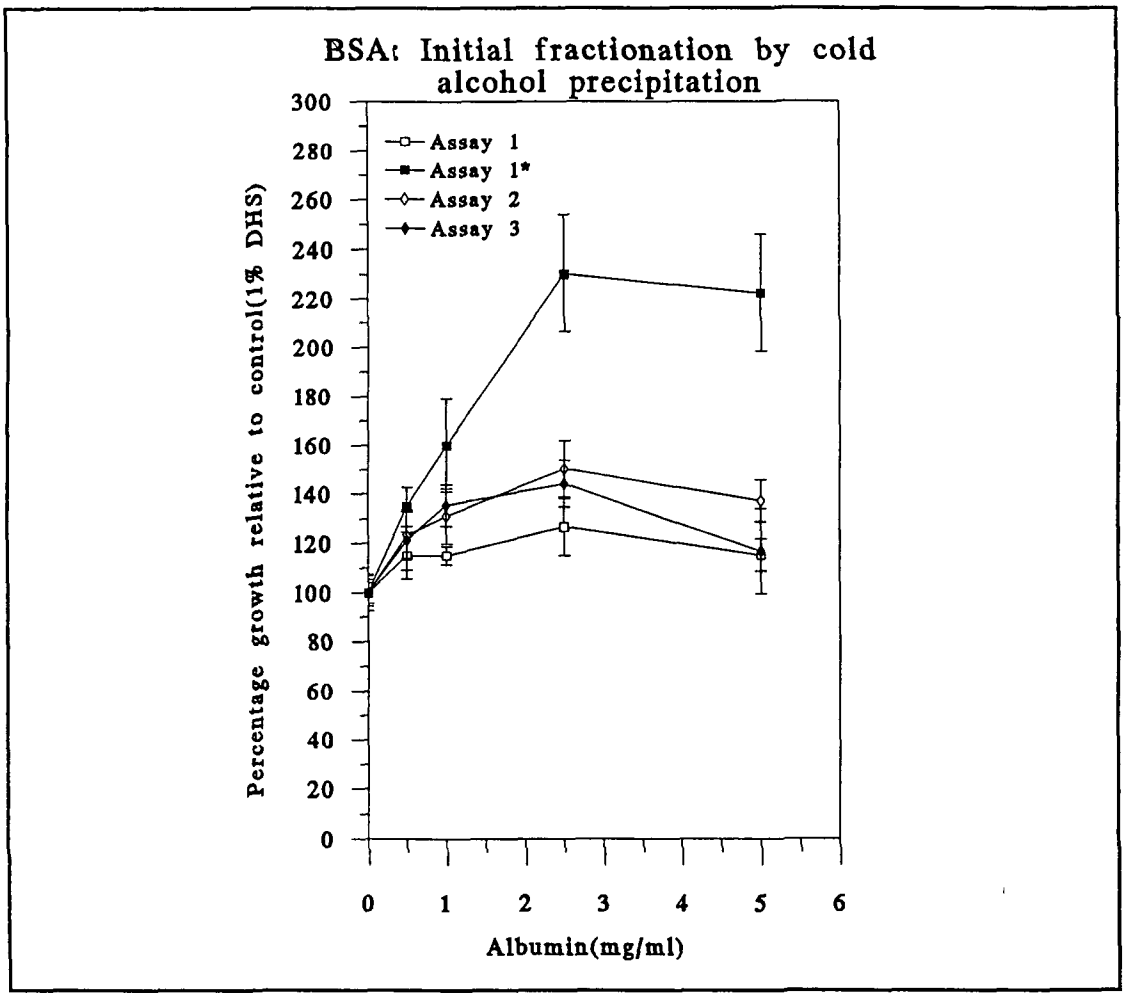


Figure 3 5.2.4.1 shows the growth response of NRK cells to BSA with an initial fractionation step by cold alcohol precipitation Results are expressed as the average percentage growth relative to the control (1% DHS) ± standard deviation (n=8) Results were taken down using acid phosphatase as the end point except Assay 1*, which was identical to Assay 1 but it was taken down using dye elution Table 3 5 2 4 1 below shows the results for three separate experiments Abbreviations A = mg/ml Albumin

Table 3.5.2.4.1 Albumin prepared by Cold Alcohol Precipiation (Sigma A4503)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 7 49	100 0 ± 7 09	100 0 ± 5 44	100 0 ± 4 13
+ 0 5mg/ml A	115 1 ± 9 43	134 9 ± 7 93	123 4 ± 9 54	121 4 ± 12 2
+ 1 0mg/ml A	115 1 ± 3 77	160 0 ± 19 1	131 1 ± 11 1	135 4 ± 8 35
+ 2 5mg/ml A	126 7 ± 11 7	230 2 ± 23 8	150 3 ± 11 6	144 2 ± 9 48
+ 5 0mg/ml A	115 1 ± 6 60	222 2 ± 23 8	137 1 ± 8 50	116 7 ± 17 1

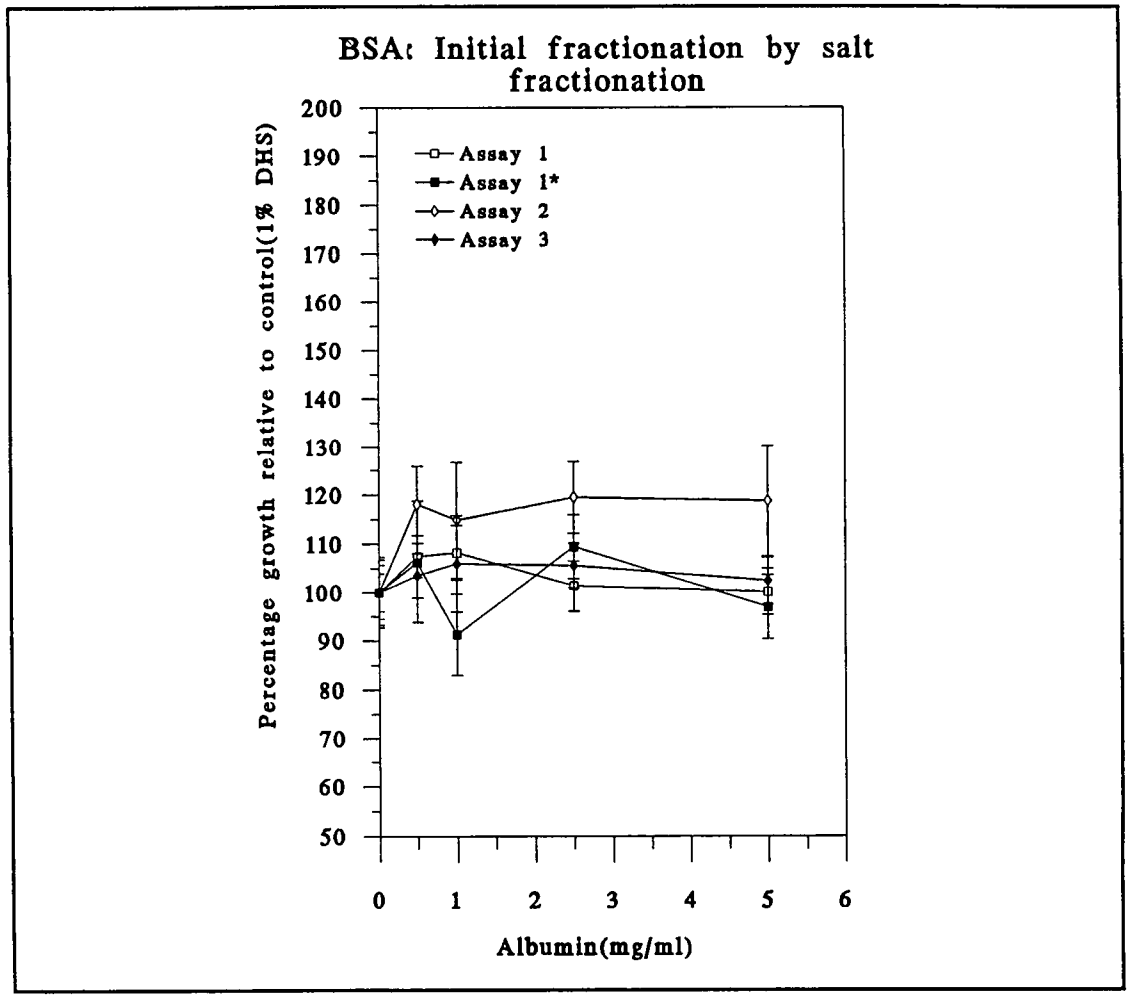


Figure 3.5.2.4 2 shows the growth response of NRK cells to BSA with an initial fractionation step by salt fractionation Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results were taken down using acid phosphatase as the end point except Assay 1* This assay was identical to Assay 1 but it was taken down by dye elution Table 3 5 2 4 2 shows the results for 3 separate assays Abbreviations A = mg/ml Albumin

Table 3.5.2.4.2 Albumin prepared by Salt Fractionation (Sigma A3675)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 3 92	100 0 ± 5 59	100 0 ± 7 35	100 0 ± 6 81
+ 0 5mg/ml A	107 4 ± 4 33	106 2 ± 12 5	118 0 ± 7 91	103 5 ± 4 61
+ 1 0mg/ml A	108 2 ± 5 63	91 25 ± 8 38	114 9 ± 11 9	105 9 ± 9 91
+ 2 5mg/ml A	101 3 ± 5 19	109 4 ± 6 55	119 5 ± 7 34	105 5 ± 4 61
+ 5 0mg/ml A	100 0 ± 4 76	96 87 ± 6 55	118 6 ± 11 3	102 3 ± 4 84

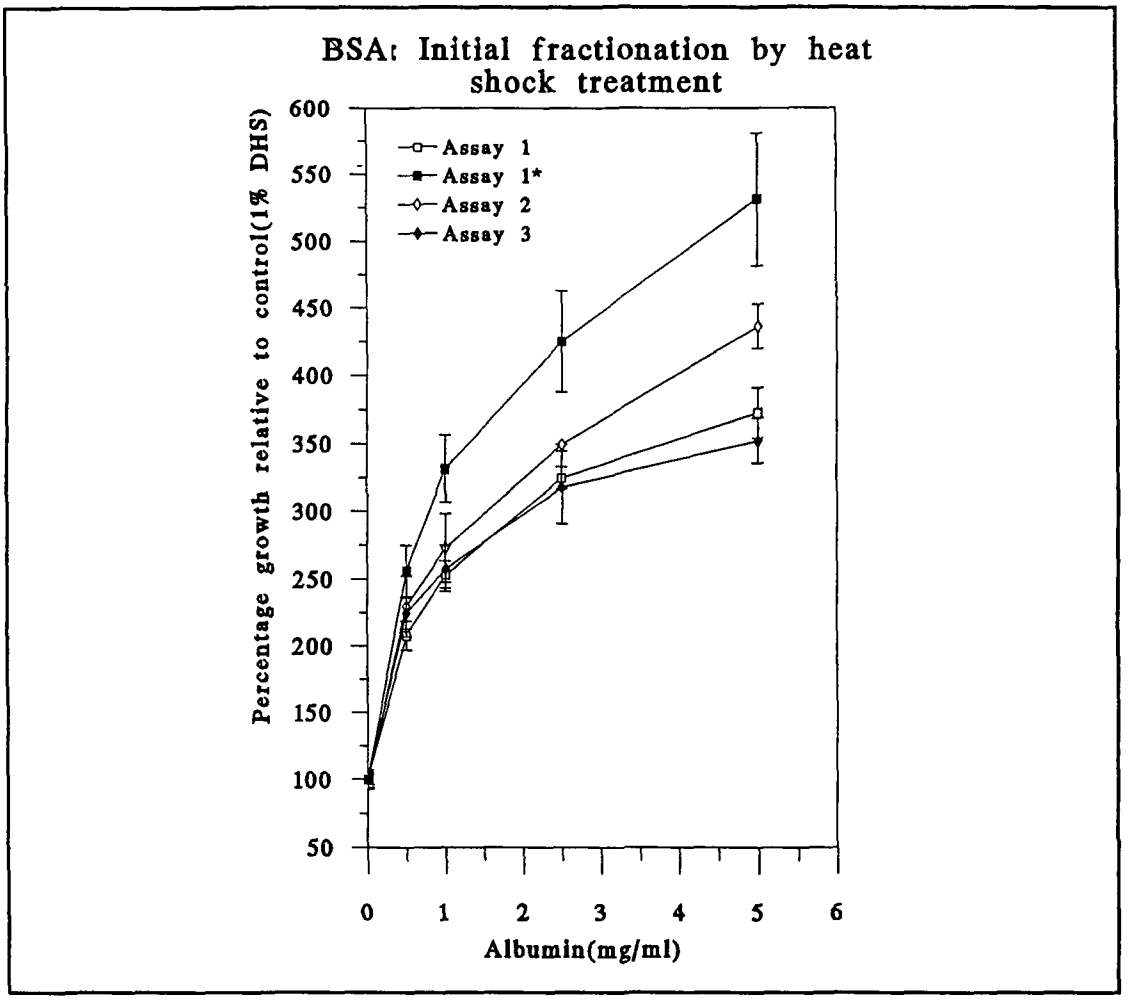


Figure 3.5.2.4.3 shows the growth response of NRK cells to BSA with an initial fractionation step by heat shock treatment Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results were taken down using acid phosphatase as the end point except Assay 1* This assay was identical to Assay 1 but it was taken down by dye elution. Table 3 5 2 4 3 shows the results for 3 separate assays Abbreviations A = mg/ml Albumin

Table 3.5.2.4.3 Albumin prepared by Heat Shock treatment (Sigma A7409)

VARIABLES	ASSAY 1	ASSAY 1*	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 3 92	100 0 ± 5 59	100 0 ± 7 35	100 0 ± 6 81
+ 0 5mg/ml A	207 8 ± 10 8	256 2 ± 18 7	229 7 ± 22 6	224 6 ± 11 7
+ 1 0mg/ml A	253 2 ± 9 99	331 2 ± 25 0	272 8 ± 25 1	257 6 ± 17 1
+ 2 5mg/ml A	324 7 ± 8 22	425 0 ± 37 5	349 4 ± 0 11	317 7 ± 26 9
+ 5 0mg/ml A	372 3 ± 18 2	531 1 ± 50 0	435 9 ± 16 4	351 8 ± 16 6

3.5.3 EXTRACTION OF LIPIDS FROM BSA USING ORGANIC SOLVENTS

Organic solvents with different dielectric constants were used to try and isolate the activity associated with BSA The method used was based on the experiments carried out by Tigyi and Miledi (1992), in which they used methanol to extract lysophospholipids from human serum albumin

The following organic solvents were used (in order of decreasing dielectric constants) methanol (MeOH), ethanol (EtOH), acetone (ACE), chloroform ($CHCl_3$), and di-ethyl-ether (DEE) In total, extraction with each solvent was carried out three times in succession The protein profile and biological activity for two or three separate experiments are shown in Tables 3 5 3 and 3 5 3 1 to 5 The biological activity was assayed two or three times for each extraction Assays were set up in 96- or 24-well plates and taken down with crystal violet dye elution or cell counts as the end points respectively All fractions were assayed at 5mg/ml protein final concentration

The protein profiles of the organic and protein phases after extraction are shown in Table 3 5 3 For all solvents used except chloroform and sometimes with acetone, a high recovery of protein in the protein phase (aqueous phase) was achieved When chloroform was used, the protein settled like a scum on top of the solvent (presumably due to denaturation) As a result, complete separation of the two phases was very difficult Very low recovery of protein using acetone in the first extraction occurred due to loss of protein when evaporating off acetone under a stream of nitrogen

Table 3.5.3 Protein Profile

Solvent	EXPERIMENT 1				EXPERIMENT 2			
	Initial Protein (mg)	Protein conc (mg) Aqueous phase	Protein conc (mg) Organic phase	Percentage recovery	Initial Protein (mg)	Protein conc (mg) Aqueous phase	Protein conc (mg) Organic phase	Percentage recovery
MeOH	108 3	91 39	≤ 0	84 38	109 4	87 62	≤ 0	80 09
EtOH	105 2	86 36	≤ 0	82 08	108 9	81 02	≤ 0	74 44
ACE	104 7	34 90	≤ 0	33 33	107 0	90 60	≤ 0	84 67
$CHCl_3$	104 4	70 88	2 25	70 04	107 4	49 66	3 1	57 80
DEE	104 7	143 2	≤ 0	136 8	109 4	83 72	≤ 0	75 43

Note The dielectric constant for each of the organic solvents are 32 6, 24 3, 20 7, 4 8 and 4 3 for methanol, ethanol, acetone, chloroform and di-ethyl-ether (Tigyi and Miledi, 1992) The protein concentration was determined by spectrophotometric absorbance at 280nm

3.5.3.1 Methanol

The growth stimulating effect of BSA after methanol extraction is shown in Table 3 5 3 1a and b The methanol control was slightly stimulatory in experiment 1 but not in experiment 2 With the organic phase, no activity was seen in experiment 1 but varying stimulation in experiment 2 (25 and 77% stimulation above the control respectively) Of the activity associated with the BSA control, only 32 7 - 40 5% was retained in the protein phase after the first experiment and 82 - 83 9% after the second experiment Combination of the organic and protein phases showed similar stimulation to that of the protein phase alone, but both were less active than the BSA control in the first experiment and almost as active as the control in the second experiment

Comparison of the activity in the protein phase to the percentage of protein recovered, shows that 84% and 80 1% protein was recovered in the two experiments respectively This could explain the loss of activity relative to the untreated BSA control in the second experiment but not in the first experiment

Table 3.5.3.1a Results for extraction with methanol

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 7 26	100 0 ± 8 41	100 0 ± 6 52	100 0 ± 9 22
+ BSA Con	283 3 ± 20 0	364 2 ± 39 7	341 0 ± 30 2	338 4 ± 17 1
+ MeOH Con	110 0 ± 12 0	135 0 ± 10 0	98 81 ± 10 9	109 2 ± 10 1
+ MeOH P	160 0 ± 16 7	207 1 ± 23 3	297 6 ± 25 9	300 0 ± 16 3
+ MeOH OP	100 0 ± 10 0	127 5 ± 14 4	125 0 ± 14 3	177 5 ± 12 2
+ MeOH POP	156 7 ± 10 0	235 1 ± 30 0	345 2 ± 19 8	295 9 ± 26 5

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Abbreviations MeOH con = solvent control without albumin, dried under stream of nitrogen and reconstituted in medium, P = protein phase of the extraction, OP = organic phase of the extraction, POP = combination of organic and protein phases after extraction process, BSA con = 5mg/ml untreated BSA

As some activity was seen in the organic phase using dye elution as the end point, the experiment was repeated This time however, cell number was used as the end point The results are shown in Table 3 5 3 1b Most or all of the activity was retained within the protein phase (106 9 and 85 5% relative to the BSA control), with little or no activity seen in the organic phase Combination of the organic and protein phase did not result in increased activity (reduction in activity in the first assay and no change in the second assay) The BSA stirring control, which was exposed to 3 x 1 hour stirring at room temperature, showed no growth at

all with either assay Although some cells appeared to have attached, no cell spreading occurred In addition no increase in cell number was detected in the supernatant The fact that some cells attached but did not spread, would indicate that the albumin in some way prevented or inhibited the laying down of an extracellular matrix

Table 3.5.3.1b Results for extraction with methanol

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 12 3	100 0 ± 10 6
+ BSA Con	226 1 ± 26 4	282 0 ± 28 3
+ BSA stirr con	0 00 ± 0 00	0 00 ± 0 00
+ MeOH Con	130 4 ± 18 4	80 50 ± 4 80
+ MeOH P	234 8 ± 43 5	255 5 ± 16 4
+ MeOH OP	118 8 ± 15 3	82 03 ± 6 63
+ MeOH POP	213 0 ± 41 4	255 5 ± 21 5

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3) Cell number was used as the end point Abbreviations as before, BSA stirr con = untreated BSA exposed to 3 x 1Hr stirrings at room temperature, also tested at 5mg/ml

3.5.3.2 Ethanol

The effect of ethanol extractions on the growth stimulating ability of BSA are shown in Table 3 5 3 2 The ethanol control showed 20 to 70% stimulation above the control (1% DHS) in experiment 1 but no stimulation in experiment 2 The organic phase showed no activity in the first experiment when the standard deviations were taken into account In the second experiment, varying inhibition was observed The amount of growth stimulating activity relative to the untreated BSA was low in the protein phase of the first experiment (47 8 and 65 6% for assays 1 and 2 respectively) and mixed in the second experiment (68 4 and 109 7% for assays 1 and 2 respectively) The combination of the organic and protein phase was slightly less stimulatory than the protein phase alone (in 3 of 4 assays)

The percentage protein recovered after each experiment could account for some of the loss in stimulation relative to the untreated BSA in the first experiment, 82% protein recovered and 74 4% protein recovery in the second experiment

Table 3.5.3.2 Results for extraction with ethanol

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 7 26	100 0 ± 8 41	100 0 ± 6 52	100 0 ± 9 22
+ BSA Con	283 3 ± 20 0	364 2 ± 39 7	341 0 ± 30 2	338 4 ± 17 1
+ EtOH Con	120 0 ± 10 0	170 0 ± 15 0	91 40 ± 5 40	97 66 ± 6 20
+ EtOH P	187 6 ± 10 9	273 3 ± 19 4	264 8 ± 2 46	361 5 ± 35 6
+ EtOH OP	113 3 ± 10 0	111 7 ± 8 16	85 12 ± 10 4	57 43 ± 16 2
+ EtOH POP	160 9 ± 7 14	220 0 ± 20 0	277 9 ± 22 9	349 8 ± 18 4

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Abbreviations as before

3.5.3.3 Acetone

The effect of acetone extraction on the growth stimulatory activity of BSA is shown in Table 3 5 3 3 The organic phase alone showed no stimulation when the acetone controls were taken into account In the first experiment, only 12 6 and 22 6% of the activity seen with the control BSA remained with the protein phase However, only 33% of the protein was recovered For the second experiment, 50 7 and 56 6% of the activity remained with the protein phase In this experiment, 84 7% of the protein was recovered However, the combination of the organic and protein phases showed more stimulation than the protein phase alone The results suggest that some activity had been isolated in the organic phase and that on recombination of the organic and protein phase, the effect was greater than their additive stimulation in 3 of 4 assays However, the amount of activity in the protein or the combined protein and organic phase was lower than the untreated BSA (57 8 and 45 6% in the first experiment, 30 2 and 75% in the second experiment)

Table 3 5.3.3 Results for extraction with acetone

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 7 26	100 0 ± 8 41	100 0 ± 6 52	100 0 ± 9 22
+ BSA Con	407 1 ± 45 1	376 6 ± 24 4	341 0 ± 30 2	356 0 ± 19 8
+ Ace con	99 96 ± 12 2	137 4 ± 8 15	73 47 ± 14 1	97 18 ± 7 40
+ Ace P	138 8 ± 20 7	162 5 ± 22 6	222 3 ± 36 2	245 0 ± 19 3
+ Ace OP	114 3 ± 12 6	128 8 ± 12 8	71 43 ± 17 1	98 60 ± 7 40
+ Ace POP	277 6 ± 23 4	226 2 ± 20 4	172 8 ± 36 4	295 9 ± 26 5

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Abbreviations as before

3.5.3.4 Chloroform

The effect of chloroform extraction on the growth stimulating activity of BSA is shown in Table 3 5 3 4 The chloroform control showed inhibition in 3 of 4 bioassays The protein phase had little activity associated with it in the first experiment which may be related to the extent of protein recovered after the extraction Good stimulation was seen in the second experiment The organic phase was about 25% inhibitory in 3 of 4 bioassays Synergism was seen in the combination of the organic and protein phase in the first experiment but not in the second experiment The extent of activity (relative to the untreated BSA) recovered in the recombination was 46 and 40% (assay 1 and assay 2) for the first experiment and 65 6 and 58 4% for the second experiment For the protein phase alone, the activity was 5 36 and 25% (assay 1 and assay 2) in the first experiment and 107 8 and 63 6% (assay 1 and 2) in the second experiment The amount of protein recovered was 67 9% and 46 2% for experiments 1 and 2 respectively So, in the first experiment, the extraction process appeared to destroy 60% of the activity while in the second experiment, 40% activity was destroyed

Table 3.5.3 4 Results for extraction with chloroform

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 7 26	100 0 ± 8 41	100 0 ± 6 52	100 0 ± 9 22
+ BSA Con	231 3 ± 24 2	348 3 ± 31 0	387 5 ± 30 8	308 1 ± 52 9
+ $CHCl_3$ con	103 7 ± 7 14	86 21 ± 10 3	75 00 ± 9 37	72 97 ± 14 5
+ $CHCl_3$ P	107 4 ± 11 7	162 1 ± 13 3	410 0 ± 5 81	232 4 ± 13 5
+ $CHCl_3$ OP	74 23 ± 5 42	75 86 ± 4 88	132 5 ± 11 8	76 20 ± 12 7
+$CHCl_3$ POP	160 4 ± 11 8	199 4 ± 15 3	288 7 ± 42 5	221 6 ± 3 52

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Abbreviations as before

3.5.3.5 Di-ethyl-ether

The effect of extraction using di-ethyl-ether (DEE) on the growth stimulating activity of BSA is shown in Table 3 5 3 5a and b The DEE control showed 10 to 27% inhibition in the two experiments The organic phase showed inhibition also In three of four assays, addition of the organic and protein phase did not produce higher activity than that seen with the protein phase alone In the first experiment, the activity seen in the protein phase relative to the BSA control was 67 6 and 54 6% while in the second experiment the activity was 97 8 and 70 4%

for assays one and two respectively The amount of protein recovered after the experiments was 136 8% and 83 7% for the first and second experiments respectively

Table 3.5.3.5a Results for extraction with DEE

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 7 26	100 0 ± 8 41	100 0 ± 6 52	100 0 ± 9 22
+ BSA Con	231 3 ± 24 2	348 3 ± 31 0	387 5 ± 30 8	308 1 ± 52 9
+ DEE con	73 62 ± 7 36	89 65 ± 10 3	68 75 ± 6 25	74 90 ± 16 4
+ DEE P	188 8 ± 14 4	235 6 ± 20 1	381 2 ± 37 5	246 5 ± 35 4
+ DEE OP	84 05 ± 7 14	105 2 ± 15 5	92 50 ± 11 2	66 22 ± 6 91
+ DEE POP	180 6 ± 22 2	290 8 ± 25 3	288 7 ± 40 0	186 9 ± 26 2

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Abbreviations as before

An additional experiment was carried out using cell number as the end point Again most of the activity was retained in the protein phase (107 3 and 73 6% relative to the untreated BSA, for duplicate repeats) The addition of the organic phase (no activity alone) resulted in different trends, assay 2 showed a small increase and assay 1 showed a decrease From the three experiments overall, the addition of the organic phase to the protein phase resulted in a decrease in activity

Table 3.5.3.5b Results for extraction with DEE

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 6 64	100 0 ± 12 5
+ BSA Con	260 0 ± 44 2	279 2 ± 22 0
+ BSA stirr con	0 00 ± 0 00	241 7 ± 19 1
+ DEE Con	113 2 ± 9 80	79 17 ± 12 5
+ DEE P	271 7 ± 11 3	231 9 ± 34 7
+ DEE OP	88 68 ± 11 8	93 05 ± 9 62
+ DEE POP	224 5 ± 31 2	283 3 ± 31 5

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3) Cell number was used as the end point Abbreviations as before

3.5.3.6 Addition of LPA/PA to inactive albumin

Another route of investigation to determine the possible role of lysophosphatidic (LPA) or phosphatidic (PA) acid in the stimulation of NRK cells by BSA was to add LPA or PA to inactive albumin to see if activity could be restored

Based on the work of Moolenaar *et al* (1986) and Tigyi and Miledi (1992) concentrations of 0 1, 1 0, 5 0 and 10 0μg/ml LPA/PA were chosen Assays were set up with LPA/PA with and without inactive albumin The unbound fraction obtained upon heparin sepharose treatment of BSA fraction V was chosen as the inactive albumin (see section 3 5 4) The results are shown in Table 3 5 3 6a and b The results show that LPA or PA alone or in combination with the unbound fraction did not affect cell growth

Table 3.5.3.6a Effect of phosphatidic acid on NRK cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 5 39	100 0 ± 6 82	100 0 ± 7 73
+ 0 1μg/ml PA	98 83 ± 8 63	100 5 ± 9 33	104 2 ± 6 71
+ 1 0μg/ml PA	101 1 ± 10 5	108 5 ± 14 0	105 7 ± 6 32
+ 5 0μg/ml PA	99 79 ± 7 04	101 4 ± 14 1	101 0 ± 6 34
+ 10 0μg/ml PA	109 4 ± 13 4	109 6 ± 16 8	106 0 ± 6 16
+ 2mg/ml Unbound Fr	105 2 ± 9 27	115 4 ± 12 2	97 89 ± 8 52
+ 0 1μg/ml PA	104 6 ± 8 98	110 6 ± 5 24	90 32 ± 7 89
+ 1 0μg/ml PA	104 9 ± 12 5	111 1 ± 7 87	89 01 ± 2 60
+ 5 0μg/ml PA	92 98 ± 4 87	118 3 ± 9 22	90 55 ± 4 50
+ 10 0μg/ml PA	96 54 ± 4 46	91 86 ± 6 54	106 8 ± 5 68

Results are given as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Results for three separate assays are shown here Abbreviations PA = Dioleoyl L-α-phosphatidic acid (Cat No P2767), Unbound Fr = unbound fraction obtained during purification of BSA on a heparin sepharose column (see section 3 5 4)

Table 3.5.3.6b Effect of lysophosphatidic acid on NRK cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 7 43	100 0 ± 6 29	100 0 ± 6 73
+ 0 1μg/ml LPA	97 50 ± 8 89	98 25 ± 8 39	101 4 ± 8 65
+ 1 0μg/ml LPA	96 67 ± 8 89	104 2 ± 6 99	102 9 ± 10 1
+ 5 0μg/ml LPA	94 44 ± 8 33	105 2 ± 4 89	102 9 ± 6 73
+ 10 0μg/ml LPA	88 61 ± 6 94	105 2 ± 8 04	113 9 ± 10 1
+ 2mg/ml Unbound Fr	96 11 ± 5 00	102 4 ± 5 94	101 4 ± 8 17
+ 0 1μg/ml LPA	76 94 ± 4 44	83 57 ± 4 89	99 59 ± 6 09
+ 1 0μg/ml LPA	78 61 ± 5 83	86 01 ± 6 99	98 14 ± 7 36
+ 5 0μg/ml LPA	76 11 ± 5 55	84 96 ± 6 64	92 79 ± 6 73
+ 10 0μg/ml LPA	73 11 ± 6 39	85 31 ± 9 09	95 33 ± 7 86

Results are given as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point Results for three separate assays are shown here Abbreviations LPA = Oleoyl L-α-lysophosphatidic acid (Cat No L7260), Unbound Fr = unbound fraction obtained during purification of BSA on a heparin sepharose column (see section 3 5 4)

Taken together, the results of sections 3 5 3 1-6 would indicate, that some of the activity associated with BSA was lost upon a triple extraction with methanol, ethanol, acetone, chloroform or di-ethyl-ether Little or no activity was associated with the organic phase Recombination of the respective organic and protein phases only resulted in a synergistic effect for acetone and in one of the two extractions with chloroform It appeared that the extraction process resulted in the destruction of some of the activity and could not be restored on recombination of the organic and protein phases

As some of the activity appeared to have been lost during the extraction process, another means of testing the possible role of the phosphatides and lysophosphatides was taken The growth stimulating effect of lysophosphatidic and phosphatidic acid were investigated both in the presence and absence of inactive albumin The results in Table 3 5 3 6a and b showed that neither lysophosphatidic nor phosphatidic acid alone or in combination with inactive albumin could display the activity seen in untreated BSA at the concentrations tested

These results suggest that the active component associated with albumin for NRK cells was not lysophosphatidic or phosphatidic acid However some stimulatory activity was removed by extraction with acetone

3.5.4 HEPARIN SEPHAROSE CHROMATOGRAPHY OF BSA

Heparin Sepharose (HS) was used as a means of trying to isolate some of the activity associated with bovine serum albumin (BSA) The method is described in Section 2 13 Briefly, BSA fraction V was mixed HS matrix The unbound albumin was collected The column was then sequentially washed with 50mM Na_2HPO_4 buffer, 0 5M NaCl, 1 0M NaCl and 2 0M NaCl (all in 50mM Na_2HPO_4 at pH 7 4) All fractions and the control BSA sample (the remainder of the BSA in 50mM Na_2HPO_4 not added to the column) were then diafiltered against ATCC medium using diafiltration membranes with 3,000 and 5,000 molecular weight cut off points (YM3 and YM5) All fractionated fractions were adjusted to the same starting volume of the BSA solution applied to the column and subsequently referred to as albumin (equivalent) concentration This was done in order to compare the biological activities Protein concentrations were determined before dilution into ATCC medium, by measuring the absorbance at 280nm with a 50mM Na_2HPO_4 blank used as a zero at 600nm The protein concentration of the BSA control was used to calculate the dilutions of **all** fractions Aliquots were assayed for biological activity on the basis of volume (not actual protein concentration) All fractions were concentrated to the same volume as the untreated BSA control So a factor which may have been isolated from the albumin would be at the concentration of the factor present in the unfractionated albumin For this reason, all dilutions were referred to albumin equivalent concentrations The bioactivity of the fractions was checked on NRK cells which had shown growth response to BSA (section 3 5 1) Table 3 5 4 1 1 shows the protein concentration in the fractions The same BSA fraction V was used for all heparin sepharose work (Sigma A4919)

3.5.4.1 Comparison of activity and protein profiles

Of the total protein recovered from the column, most was found in the unbound fraction, between 60% - 70% for the smaller scale columns and 78 - 86% for large-scale columns (Table 3 5 4 1 2) The exception was in run 4, where approx 90% was recovered in the unbound (Note, the loss of a reading for the protein level in the buffer wash, may explain the discrepancy in these trends) The remaining protein was eluted during the buffer wash, with only trace amounts seen in the 0 5M NaCl wash and none detected in the 1 0 or 2 0M NaCl washes In larger scale preparations used in conjunction with the Biopilot, a similar trend was observed About 78 - 86% was recovered in the unbound fraction, approximately 10% in the buffer wash and 2-3% in the 0 5M NaCl wash, except for runs 9 and 10 where the amount of protein recovered in the buffer was higher (15 8 to 19 5% protein recovered) than in previous

Table 3.5.4.1.1 Protein concentration (mg/ml)

FRACTION	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl	1M, 2M NaCl	INITIAL BSA
Run 1	9 265	3 796	0 237	≤ 0	14 390
Run 2	7 826	4 318	N D	≤ 0	13 220
Run 3	8 590	3 65	0 359	≤ 0	15 697
Run 4	14 12	---	1 484	≤ 0	15 652
Run 5	26 27	3 058	0 990	≤ 0	14 213
Run 6	22 76	2 6	1 138	≤ 0	10 708
Run 7	20 91	2 654	0 869	≤ 0	11 818
Run 8	24 23	3 220	1 113	≤ 0	14 363
Run 9	23 38	4 560	0 804	≤ 0	15 100
Run 10	22 28	5 580	0 750	≤ 0	14 680

Results show the protein concentration (mg/ml) for each of the fractions for 10 separate HS fractionations The concentration of the BSA control is also shown Runs 1 to 4 were carried out using small columns of about 15ml matrix 18ml of a 15mg/ml BSA sample was applied to the column (except Run 3, only 15ml applied) Fractions were collected and concentrated down to 18ml (15ml for Run 3) Runs 5 to 10 represent six fractionations carried out with a large columns containing approximately 40ml matrix, in conjunction with the Biopilot The starting volume of each run was 53ml of a 15mg/ml BSA solution The final volume of each fraction was 25ml

Table 3.5.4.1.2 Protein distribution (based on percent of protein recovered)

FRACTION	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl	1M,2M NaCl	% REC
Run 1	69 67	28 54	1 78	≤ 0	92 41
Run 2	64 44	35 50	N D	≤ 0	91 86
Run 3	68 23	28 91	2 86	≤ 0	96 32
Run 4	90 50	---	9 90	≤ 0	99 88
Run 5	86 65	10 09	3 25	≤ 0	100 60
Run 6	85 89	9 81	4 29	≤ 0	93 38
Run 7	85 58	10 86	3 56	≤ 0	97 54
Run 8	84 83	11 27	3 89	≤ 0	93 80
Run 9	81 34	15 86	2 79	≤ 0	89 45
Run 10	77 75	19 49	2 62	≤ 0	92 07

Results show the percentage protein distribution for the fractions from 10 runs with the initial BSA concentration Abbreviations % REC = amount of protein in fractions as a percentage of the total amount of protein added to the column

runs on the biopilot The total recovery of protein as a percentage of the amount applied was greater than 90% recovery on both small and large-scale fractionations

Note It was necessary to establish that diafiltration did not result in a loss of activity This was shown to be the case for NRK cells using membranes with 1,000Da and 5,000Da molecular weight cut off (Appendix I)

After diafiltering, analysis of the bioactivity of the fractions was carried out using NRK cells with a background of 1% DHS Serial dilutions with a concentration range of 0 5 - 5 0mg/ml were tested Acid phosphatase was used as the end point for runs 1 to 6 For remaining runs, dye elution from 96-well plates and cell number from 24-well plates were used as end points

When the protein concentrations were superimposed on the biological activity of the fractions, the results were very interesting (Appendix G) The maximum biological activity did not correspond to the bulk of protein recovered (Figure 3 5 4 1 3) Little growth stimulation or growth inhibition was seen in the unbound fractions and buffer washes, where greater than 95% protein was recovered The level of this inhibition varied from run to run Activity similar to that of the control albumin was seen in the 0 5M NaCl fraction in all but two runs In the first run on the Biopilot, the 0 5M NaCl fraction was nearly diafiltered dry In the third run on the smaller column, the activity was present in the 1 0M NaCl fraction Some activity was also seen in the 2 0M NaCl wash in Run 3 Figure 3 5 4 1 4 shows the typical growth response of NRK cells to dilutions of each fraction

For all fractionation processes (Runs), best stimulation of the 0 5M NaCl fraction occurred at 5mg/ml equivalent concentration This concentration was used for further analysis of the active component

Table 3 5.4.1.3 Biological activity of fractions obtained from HS chromatography on NRK cells

RUN	BSA CONTROL	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl	1M NaCl	2M NaCl
Run 1	127 7 ± 19 0	48 9 ± 3 9	72 8 ± 4 7	112 0 ± 8 90	91 7 ± 4 6	84 7 ± 7 5
Run 2	183 5 ± 12 4	98 0 ± 5 2	76 1 ± 7 9	205 0 ± 12 4	94 9 ± 5 6	89 2 ± 4 3
Run 3	157 4 ± 11 9	67 3 ± 5 4	72 7 ± 5 2	101 0 ± 6 50	150 0 ± 9 3	138 0 ± 11 8
Run 4	148 5 ± 9 70	94 7 ± 12 0	105 0 ± 4 2	169 0 ± 14 4	113 0 ± 7 3	109 0 ± 10 2
Run 5	127 1 ± 8 93	53 7 ± 1 90	75 6 ± 4 4	121 0 ± 9 40	99 9 ± 7 0	108 0 ± 6 64
Run 6	164 8 ± 9 13	92 7 ± 8 00	93 0 ± 4 2	120 0 ± 8 6	91 7 ± 5 9	91 1 ± 2 9
Run 7	205 3 ± 11 9	103 0 ± 18 1	123 0 ± 9 6	321 0 ± 35 2	91 9 ± 12 0	109 0 ± 9 67
Run 8	286 9 ± 28 3	97 0 ± 8 4	109 0 ± 6 4	177 0 ± 7 21	100 0 ± 12 8	106 0 ± 6 12
Run 9	175 0 ± 23 7	45 4 ± 18 0	79 5 ± 9 6	176 0 ± 11 2	71 2 ± 3 50	92 4 ± 12 5
Run 10	349 7 ± 26 5	73 8 ± 11 6	74 3 ± 5 0	337 0 ± 16 5	85 6 ± 5 39	-----

Results are given as the average percentage growth relative to control (1% DHS = 100%) ± standard deviation (n=8) One set of results typical of each run are shown Acid phosphatase was used as the end point for assays in runs 1 - 6 Dye elution was used as the end point for assays in runs 7 - 10 All results shown here are at 5mg/ml (equivalent) albumin concentration

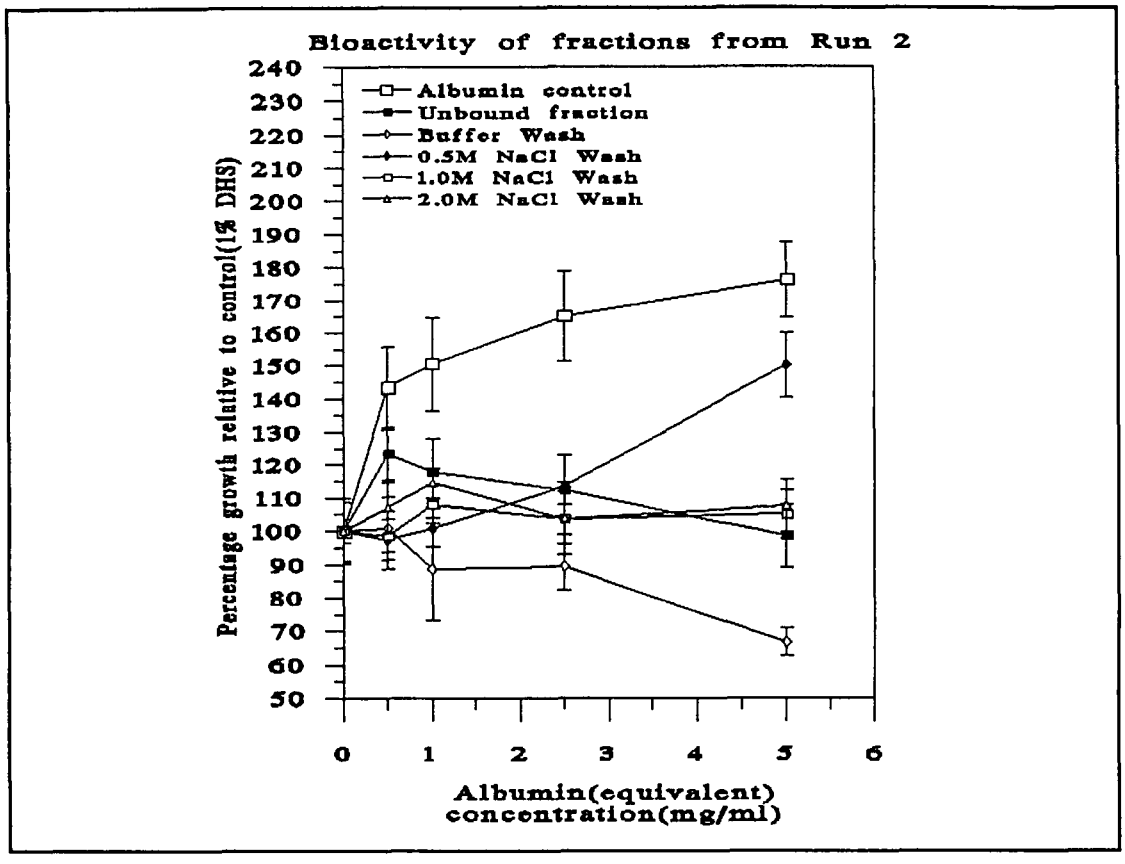
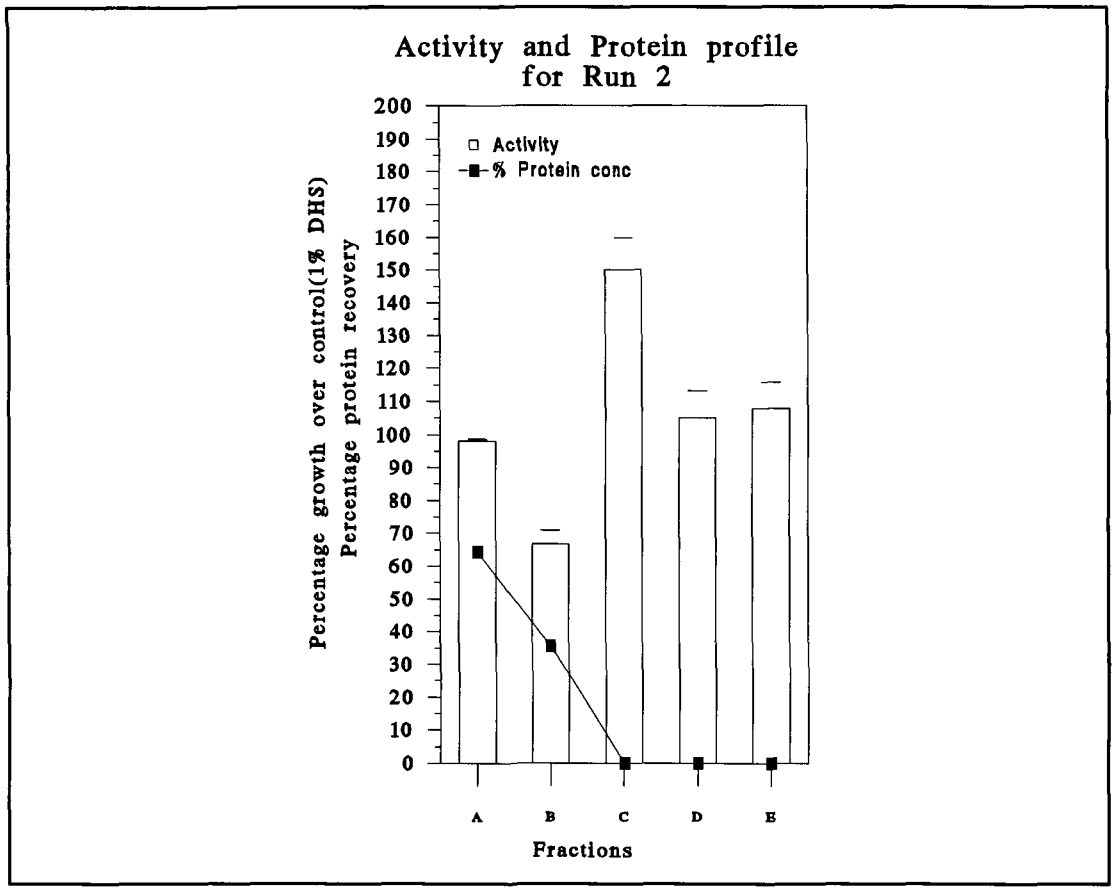


Figure 3.5.4.1.3 shows the growth response of NRK cells to a dilution curve of each fraction obtained from Run 2 (Assay 2). Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point The results for two separate assays are shown in Table 3 5 4 1 3

Table 3.5.4.1.3 Growth response of NRK cells to all the fractions at albumin(equivalent) concentration

ASSAY 1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0mg/ml	100 ± 8 76	100 0 ± 8 76	100 0 ± 9 94	100 0 ± 9 94	100 0 ± 3 60	100 0 ± 3 60
0 5mg/ml	151 ± 14 4	123 4 ± 8 11	101 0 ± 9 40	197 2 ± 8 86	98 63 ± 4 90	107 0 ± 7 80
1 0mg/ml	165 ± 13 7	117 7 ± 10 5	88 73 ± 15 2	101 0 ± 5 63	108 1 ± 5 62	114 4 ± 4 66
2 5mg/ml	176 ± 11 4	98 56 ± 9 42	89 30 ± 6 80	113 6 ± 9 10	103 6 ± 4 60	103 8 ± 10 8
5 0mg/ml	143 ± 12 4	112 6 ± 0 65	66 62 ± 4 20	150 0 ± 9 80	105 1 ± 7 40	107 7 ± 7 94
ASSAY 2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0mg/ml	100 ± 6 85	100 0 ± 6 85	100 0 ± 12 9	100 0 ± 12 9	100 0 ± 6 60	100 0 ± 6 60
0 5mg/ml	144 ± 7 40	117 8 ± 5 20	107 0 ± 10 9	121 0 ± 14 8	95 15 ± 5 30	103 0 ± 9 70
1 0mg/ml	151 ± 8 80	106 0 ± 5 70	103 3 ± 10 7	124 5 ± 11 8	92 84 ± 3 35	107 0 ± 8 31
2 5mg/ml	160 ± 14 9	98 12 ± 6 46	91 76 ± 11 5	149 9 ± 15 9	101 6 ± 6 47	102 2 ± 8 08
5 0mg/ml	184 ± 12 4	98 00 ± 5 20	76 10 ± 7 97	205 5 ± 12 4	100 9 ± 5 60	76 25 ± 8 54



Figures 3.5.4.1.4 shows the biological activity and the corresponding protein concentration of each fraction from run 2 The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were read using acid phosphatase as the end point The values for the BSA and ATCC controls are shown in Table 3 5 4 1 3 Results for biological activity are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations A = unbound fraction, B = buffer wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein concentration is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column

Table 3.5.4.1.3 shows the growth response of NRK cells to all the fractions at 5mg/ml albumin (equivalent) concentration

	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
1	100 ± 8 7	176 2 ± 11 4	98 6 ± 9 4	66 6 ± 4 2	150 ± 9 8	105 ± 7 4	108 ± 7 9
2	100 ± 6 8	183 5 ± 12 4	98 0 ± 5 2	76 1 ± 7 9	205 ± 12 4	94 9 ± 5 6	89 2 ± 4 3

3.5.4.2 Effect of salt concentration on HS Activity

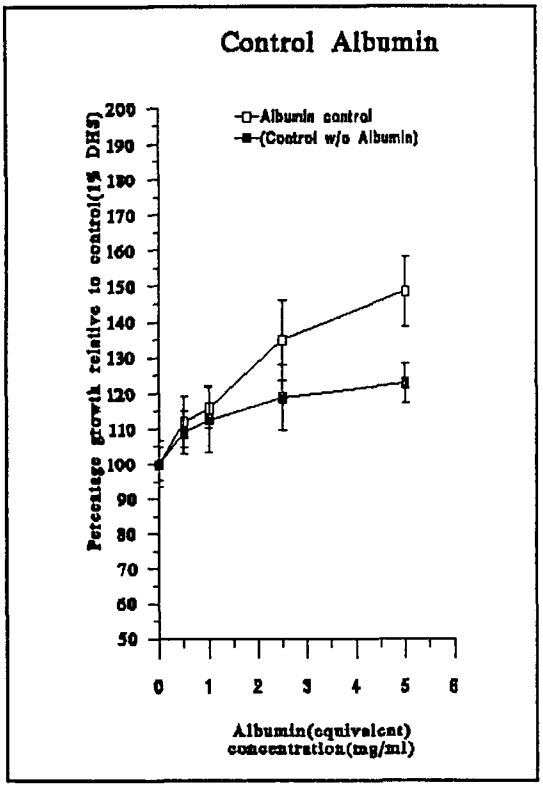
To investigate the possibility that the apparent activity in HS fractions was due to some artifact of the fractionation procedure, a second sample without albumin (50mM Na_2HPO_4 only) was run in parallel with **Run 4** (*i e* two fractionations, one with 15mg/ml BSA in 50mM Na_2HPO_4 as the starting material and the second with the buffer alone) Each sample was fractionated identically on the HS column The results are shown in Figures 3 5 4 2 1 to 3 5 4 2 6

The results showed that the major activity was eluted off in the 0 5M NaCl wash, with smaller activity in the 1 0 and 2 0M NaCl washes So, in the three runs in which activity was detected, the bulk of activity was present in the 0 5 and 1 0M NaCl washes

The salt did appear to have a slight stimulatory effect in the 1 0 and 2 0M NaCl washes (Figures 3 5 4 2 5 and 3 5 2 6), 19% and 10% stimulation over the control, respectively These results could explain the stimulatory activity seen in the 1 and 2M NaCl fractions in this run However, the extent of stimulation in Run 3 was much greater, indicating that the salt alone was not responsible for the activity

Stimulation was also seen in the unbound fraction (10 - 20%) and no inhibition in the buffer wash It appeared that the salt and the Na_2HPO_4 buffer had a slight stimulatory effect (Figures 3 5 4 2 1 and 3 5 4 2 2) In the 0 5M NaCl wash without albumin, 10% inhibition was seen (Figure 3 5 4 2 4) The activity seen with the 0 5M NaCl fraction with albumin was high The difference between the two was significant, indicating no stimulatory effect by the 0 5M NaCl alone

In conclusion for this run, the effect of the background salt concentration was found to have a slight effect for the 1 0 and 2 0M NaCl fractions No stimulation by the salt was seen in the 0 5M NaCl fraction where the majority of the activity was seen



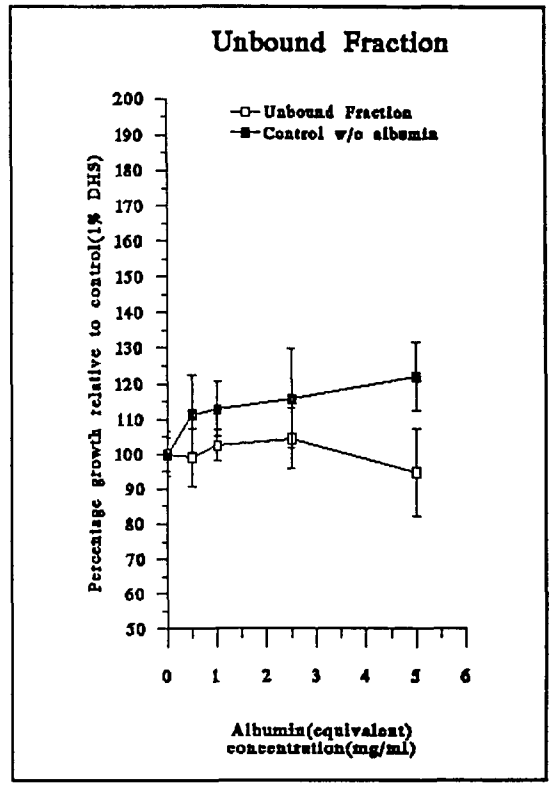


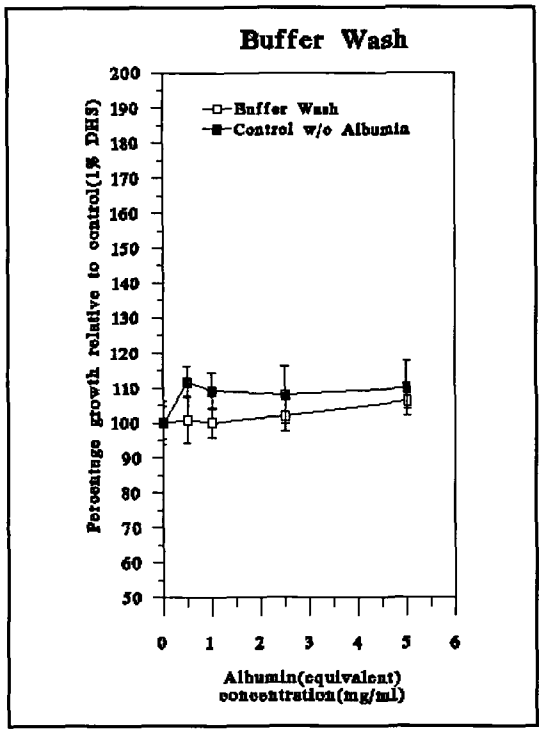
Figure 3.5.4.2.1 and 3.5.4.2.2 show the growth response of NRK cells to the control albumin and unbound fraction respectively (Assay 1) All results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results were determined using acid phosphatase as the end point Tables 3 5 4 2 1 and 3 5 4 2 2 show the results for two independent assays for the control without albumin and three for the sample with albumin Abbreviations w/o A = without albumin

Table 3.5.4.2.1 Growth response of NRK cells to the control albumin (mg/ml)

Fraction	Albumin(w/o A) Assay 1	Albumin(w/o A) Assay 2	Con Albumin Assay 1	Con Albumin Assay 2	Con Albumin Assay 3
0 0mg/ml	100 0 ± 4 90	100 0 ± 4 90	100 0 ± 6 58	100 0 ± 5 58	100 0 ± 4 46
0 5mg/ml	108 9 ± 5 95	107 0 ± 4 67	112 0 ± 7 28	116 6 ± 5 36	98 10 ± 5 72
1 0mg/ml	112 7 ± 9 40	110 3 ± 4 98	116 0 ± 5 80	120 3 ± 4 43	99 88 ± 4 40
2 5mg/ml	118 9 ± 9 20	117 4 ± 8 41	134 9 ± 11 2	124 0 ± 4 89	109 7 ± 5 08
5 0mg/ml	122 9 ± 5 50	118 7 ± 5 30	148 5 ± 9 70	129 6 ± 4 43	110 6 ± 4 24

Table 3.5.4.2 2 Growth response of NRK cells to the Unbound fractions (mg/ml)

Fraction	Unbound(w/o A) Assay 1	Unbound(w/o A) Assay 2	Unbound fraction Assay 1	Unbound fraction Assay 2	Unbound fraction Assay 3
0 0mg/ml	100 0 ± 4 90	100 0 ± 4 90	100 0 ± 6 58	100 0 ± 4 46	100 0 ± 5 58
0 5mg/ml	111 4 ± 10 9	100 9 ± 5 90	99 03 ± 8 25	92 16 ± 5 29	106 8 ± 5 13
1 0mg/ml	112 7 ± 7 71	107 2 ± 7 50	102 4 ± 4 37	92 13 ± 5 93	109 3 ± 4 43
2 5mg/ml	115 7 ± 13 9	118 1 ± 4 67	104 4 ± 8 70	86 44 ± 3 39	109 8 ± 3 73
5 0mg/ml	122 0 ± 9 70	113 4 ± 7 48	94 66 ± 12 6	74 36 ± 5 72	117 0 ± 6 30



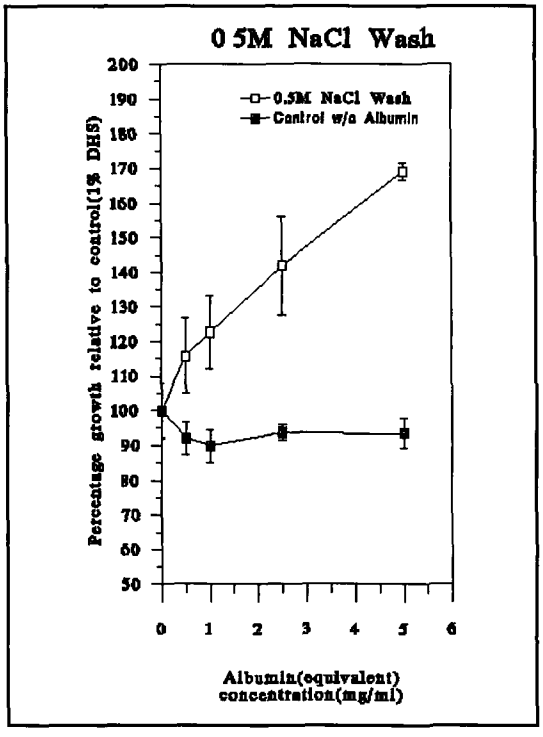


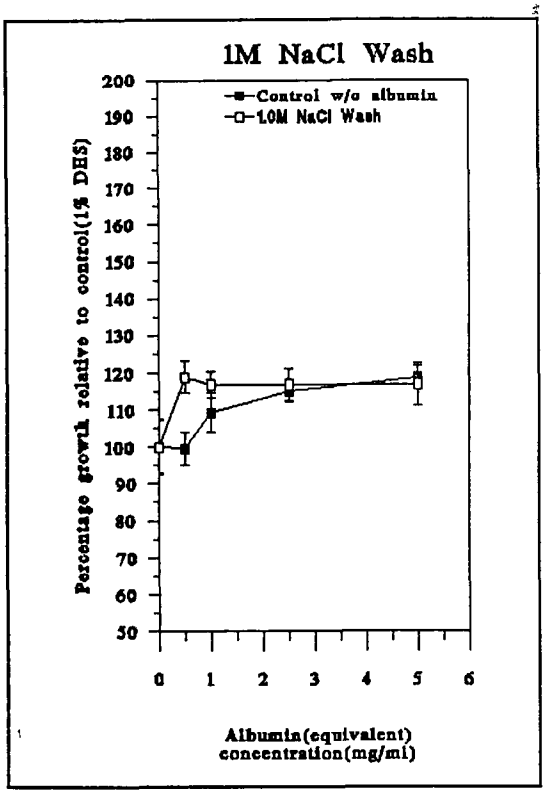
Figure 3.5.4.2.3 and 3.5.4.2.4 show the growth response of NRK cells to the buffer and 0 5M NaCl washes respectively (Assay 1) All results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results were determined using acid phosphatase as the end point Tables 3 5 4 2 3 and 3 5 4 2 4 show the results for two independent assays for the control without albumin and three for the sample with albumin Abbreviations w/o A = without albumin

Table 3.5.4.2.3 Growth response of NRK cells to the buffer wash

Fraction	Albumin(w/o A) Assay 1	Albumin(w/o A) Assay 2	Con Albumin Assay 1	Con Albumin Assay 2	Con Albumin Assay 3
0 0mg/ml	100 0 ± 6 28	100 0 ± 8 20	100 0 ± 4 49	100 0 ± 7 98	100 0 ± 6 57
0 5mg/ml	111 7 ± 4 56	111 0 ± 10 4	100 9 ± 6 63	102 0 ± 8 91	106 2 ± 7 44
1 0mg/ml	109 0 ± 5 21	110 3 ± 7 30	100 0 ± 4 24	108 0 ± 8 41	104 6 ± 6 38
2 5mg/ml	108 0 ± 8 14	114 9 ± 6 46	102 0 ± 4 42	118 0 ± 6 93	108 5 ± 6 91
5 0mg/ml	110 0 ± 7 82	110 1 ± 7 30	106 2 ± 2 39	125 0 ± 6 93	105 5 ± 5 67

Table 3.5.4.2.4 Growth response of NRK cells to the 0 5M NaCl wash

Fraction	Unbound(w/o A) Assay 1	Unbound(w/o A) Assay 2	Unbound fraction Assay 1	Unbound fraction Assay 2	Unbound fraction Assay 3
0 0mg/ml	100 0 ± 8 20	100 0 ± 6 28	100 0 ± 7 98	100 0 ± 6 57	100 0 ± 4 49
0 5mg/ml	92 13 ± 4 77	92 83 ± 3 91	115 8 ± 10 9	111 7 ± 6 91	109 8 ± 7 00
1 0mg/ml	89 89 ± 4 77	90 88 ± 4 89	122 8 ± 10 4	118 4 ± 9 75	114 4 ± 6 08
2 5mg/ml	93 82 ± 2 50	91 86 ± 3 58	141 1 ± 10 9	135 6 ± 8 51	133 0 ± 5 16
5 0mg/ml	93 54 ± 4 21	96 09 ± 4 23	169 3 ± 14 4	160 8 ± 12 4	160 3 ± 10 3



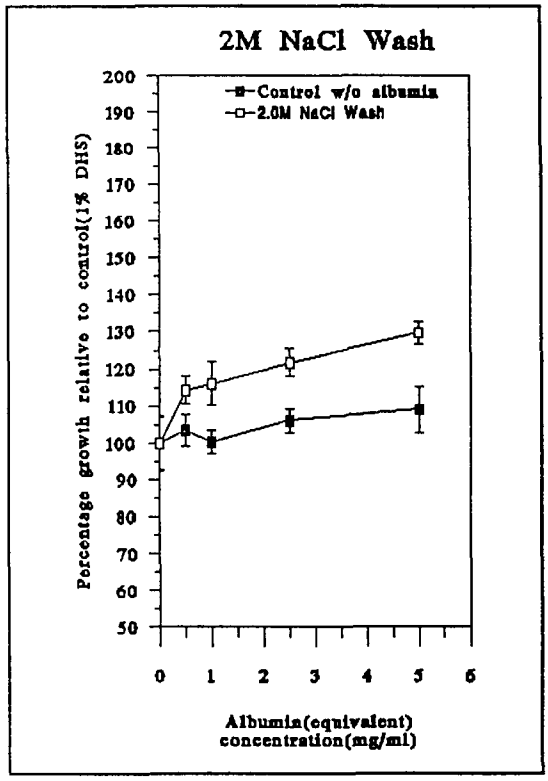


Figure 3.5.4.2.5 and 3.5.4 2.6 show the growth response of NRK cells to the 1 0 and 2 0M NaCl washes respectively (Assay 2) All results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Results were determined using acid phosphatase as the end point Tables 3 5 4 2 5 and 3 5 4 2 6 show the results for two independent assays for the control without albumin and three for the sample with albumin Abbreviations w/o A = without albumin

Table 3.5.4.2.5 Growth response of NRK cells to the 1 0M NaCl wash

Fraction	Albumin(w/o A) Assay 1	Albumin(w/o A) Assay 2	Con Albumin Assay 1	Con Albumin Assay 2	Con Albumin Assay 3
0 0mg/ml	100 0 ± 7 16	100 0 ± 8 03	100 0 ± 7 68	100 0 ± 7 37	100 0 ± 3 30
0 5mg/ml	99 70 ± 4 40	106 0 ± 4 66	104 4 ± 7 28	111 8 ± 4 28	99 66 ± 4 38
1 0mg/ml	109 3 ± 5 25	106 6 ± 7 00	104 4 ± 9 71	116 9 ± 4 60	105 2 ± 4 38
2 5mg/ml	115 2 ± 2 90	108 6 ± 8 33	110 7 ± 9 71	116 9 ± 4 28	113 3 ± 6 41
5 0mg/ml	118 7 ± 3 21	111 3 ± 7 66	113 1 ± 7 28	116 9 ± 5 70	118 8 ± 5 90

Table 3.5.4.2.6 Growth response of NRK cells to the 2 0M NaCl wash

Fraction	Unbound(w/o A) Assay 1	Unbound(w/o A) Assay 2	Unbound fraction Assay 1	Unbound fraction Assay 2	Unbound fraction Assay 3
0 0mg/ml	100 0 ± 7 16	100 0 ± 8 03	100 0 ± 7 68	100 0 ± 7 37	100 0 ± 3 30
0 5mg/ml	103 5 ± 4 40	100 0 ± 9 00	109 7 ± 8 70	114 3 ± 3 87	108 0 ± 3 03
1 0mg/ml	100 3 ± 3 21	106 6 ± 8 00	108 2 ± 7 28	116 0 ± 5 90	114 7 ± 3 20
2 5mg/ml	106 1 ± 3 21	108 6 ± 7 66	107 6 ± 10 8	121 7 ± 3 60	123 4 ± 3 65
5 0mg/ml	109 0 ± 6 12	115 0 ± 2 66	108 7 ± 10 2	129 7 ± 3 05	126 3 ± 9 61

3.5.4.3 Effect of HS fractions on different cell lines

Using fractions obtained from large-scale columns, the growth effects of HS fractions were investigated on MDCK and CHOK1 cells, in comparison to the indicator cell line, NRK cells MDCK and CHOK1 cells were grown and tested in serum-free media (SFM) (see section 3 2) while NRK cells were tested with a 1% DHS background

In the first experiment, limited activity was eluted off in the 0 5M NaCl wash (Appendix H) for NRK cells, the indicator cell line The fact that the 0 5M NaCl fraction was nearly diafiltered dry probably affected the activity The control albumin showed 44 - 65% stimulation above the control for the maximum concentration tested (5mg/ml)

Interestingly, for MDCK cells in SFM, no significant stimulation was observed with the control or any of the fractions (Figure 3 5 4 3 2) In a previous experiment (section 3 2 1 5) with MDCK cells in SFM, a maximum of 50% stimulation over control (SFM, no albumin) was obtained It may be that diafiltration of the albumin removed the active component from BSA for MDCK cells, as the BSA which had previously shown stimulation, had not been diafiltered Due to the lack of response of the MDCK cells to the BSA control, they were not tested again

For CHOK1 cells, the control albumin was totally inhibitory (Figure 3 5 4 3 3) As seen with the MDCK cells, the activity that was observed with BSA in SFM in section 3 2 2 5, may have been lost upon diafiltration It was also interesting that most of the inhibition seen with the BSA was present in the unbound fraction The inhibition seen with the 0 5M NaCl fraction did not appear as inhibitory as the BSA control until an equivalent albumin concentration of 2 5mg/ml was reached This inhibition may have been due to residual albumin in the 0 5M NaCl fraction

In a second experiment, the effect of HS fractions were tested on the growth of CHOK1 cells in serum-free medium and with NRK cells in 1% DHS background (Run 6)
For NRK cells (Figure 3 5 4 3 4), both the unbound fraction and the buffer were about 40% and 20 - 30% inhibitory respectively relative to the control (1% DHS) No activity was seen in the 1 0M NaCl fraction with up to 15% stimulation in the 2 0M NaCl fraction The maximum stimulation of the 0 5M NaCl and the BSA control were 20% and 40% at 5mg/ml and 2.5mg/ml respectively.

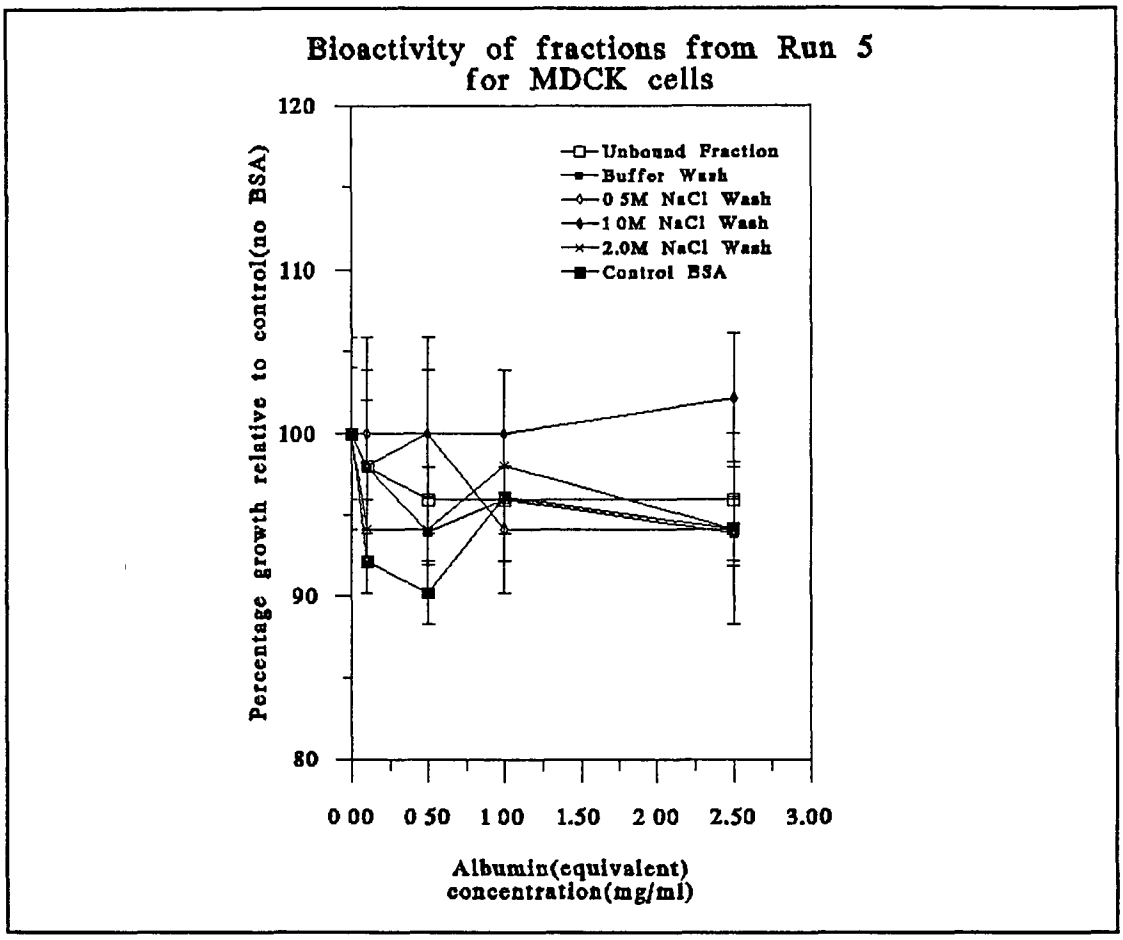


Figure 3.5.4.3.1 shows the growth response of MDCK cells to a dilution curve of each fraction obtained from Run 5 on the HS column (Assay 1) Results are expressed as the average percentage growth relative to control (SFM, no albumin) ± standard deviation (n=8) Acid phosphatase was used as the end point Results from three independent assays were are shown in Tables 3 5 4 3 1a to c

Table 3.5.4.3 1a Growth response of MDCK cells to all fractions at albumin (equivalent) concentration

ASSAY 1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 2 53	100 0 ± 3 45	100 0 ± 3 45	100 0 ± 2 32	100 0 ± 2 32	100 0 ± 2 53
0 1 mg/ml	109 ± 2 53	103 4 ± 3 45	109 2 ± 5 75	102 3 ± 3 45	105 8 ± 4 65	101 3 ± 2 53
0 5 mg/ml	104 ± 1 27	103 4 ± 4 59	106 9 ± 4 59	101 2 ± 3 45	105 8 ± 4 65	102 5 ± 2 53
1 0 mg/ml	103 ± 2 53	104 6 ± 4 59	108 0 ± 4 59	101 2 ± 3 45	106 9 ± 4 65	101 3 ± 1 27
2 5 mg/ml	103 ± 2 53	105 7 ± 4 59	106 9 ± 4 59	101 2 ± 4 65	105 8 ± 4 65	102 5 ± 2 53

Table 3.5.4.3.1b Growth response of MDCK cells to all fractions at albumin (equivalent) concentration

ASSAY 2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 5 88	100 0 ± 4 08	100 0 ± 4 08	100 0 ± 5 88	100 0 ± 5 88	100 0 ± 5 88
0 1 mg/ml	92 2 ± 1 96	97 96 ± 2 04	97 96 ± 2 04	100 0 ± 5 88	98 04 ± 5 88	94 12 ± 1 96
0 5 mg/ml	90 2 ± 1 96	95 92 ± 2 04	93 88 ± 2 04	100 0 ± 5 88	100 0 ± 3 92	94 12 ± 1 96
1 0 mg/ml	96 1 ± 3 92	95 92 ± 2 04	95 92 ± 2 04	94 12 ± 3 92	100 0 ± 3 92	98 04 ± 5 88
2 5 mg/ml	94 1 ± 1 96	95 12 ± 2 04	93 88 ± 2 04	94 12 ± 5 88	102 2 ± 3 92	94 12 ± 1 96

Table 3.5.4.3.1c Growth response of MDCK cells to all fractions at albumin (equivalent) concentration

ASSAY 3	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 5 77	100 0 ± 1 92	100 0 ± 1 92	100 0 ± 6 12	100 0 ± 6 12	100 0 ± 5 77
0 1 mg/ml	92 3 ± 3 85	96 15 ± 1 92	96 15 ± 1 92	96 50 ± 2 82	97 96 ± 4 08	94 23 ± 3 85
0 5 mg/ml	94 2 ± 1 92	94 23 ± 1 92	94 23 ± 3 85	95 92 ± 2 04	97 96 ± 4 08	94 23 ± 1 92
1 0 mg/ml	92 3 ± 3 85	96 15 ± 1 92	98 08 ± 3 85	95 92 ± 6 12	106 1 ± 4 08	94 23 ± 3 85
2 5 mg/ml	90 4 ± 1 92	94 23 ± 3 85	96 15 ± 3 85	97 96 ± 4 08	100 0 ± 4 08	94 23 ± 1 92

Table 3.5.4.3.2b Growth response of CHOK1 cells to all the fractions at albumin (equivalent) concentration

ASSAY 2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 6 71	100 0 ± 4 50	100 0 ± 4 50	100 0 ± 3 68	100 0 ± 3 68	100 0 ± 6 71
0 1 mg/ml	110 ± 7 69	117 6 ± 6 09	119 5 ± 7 70	130 0 ± 5 52	98 62 ± 4 14	104 8 ± 4 76
0 5 mg/ml	21 4 ± 1 44	57 70 ± 9 29	100 1 ± 8 09	94 50 ± 5 86	95 17 ± 5 86	91 57 ± 3 30
1 0 mg/ml	24 5 ± 1 10	16 02 ± 6 40	89 56 ± 5 22	52 24 ± 5 87	95 17 ± 6 89	89 38 ± 3 30
2 5 mg/ml	17 9 ± 0 73	14 74 ± 6 40	19 87 ± 2 56	17 24 ± 1 38	86 21 ± 5 52	78 02 ± 3 30

Table 3.5.4.3.2c Growth response of CHOK1 cells to all the fractions at albumin (equivalent) concentration

ASSAY 3	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 4 69	100 0 ± 4 51	100 0 ± 4 51	100 0 ± 6 30	100 0 ± 6 30	100 0 ± 4 69
0 1 mg/ml	99 5 ± 3 50	105 2 ± 3 64	110 0 ± 6 54	87 90 ± 3 19	93 62 ± 6 30	95 55 ± 5 53
0 5 mg/ml	60 5 ± 5 98	89 47 ± 2 91	99 31 ± 5 26	82 27 ± 3 90	102 5 ± 6 74	97 02 ± 4 06
1 0 mg/ml	34 9 ± 3 67	12 22 ± 0 71	85 54 ± 6 17	16 31 ± 0 71	91 13 ± 4 60	94 85 ± 6 38
2 5 mg/ml	11 3 ± 3 75	4 410 ± 0 13	29 17 ± 1 81	17 35 ± 0 71	75 53 ± 3 90	91 02 ± 4 91

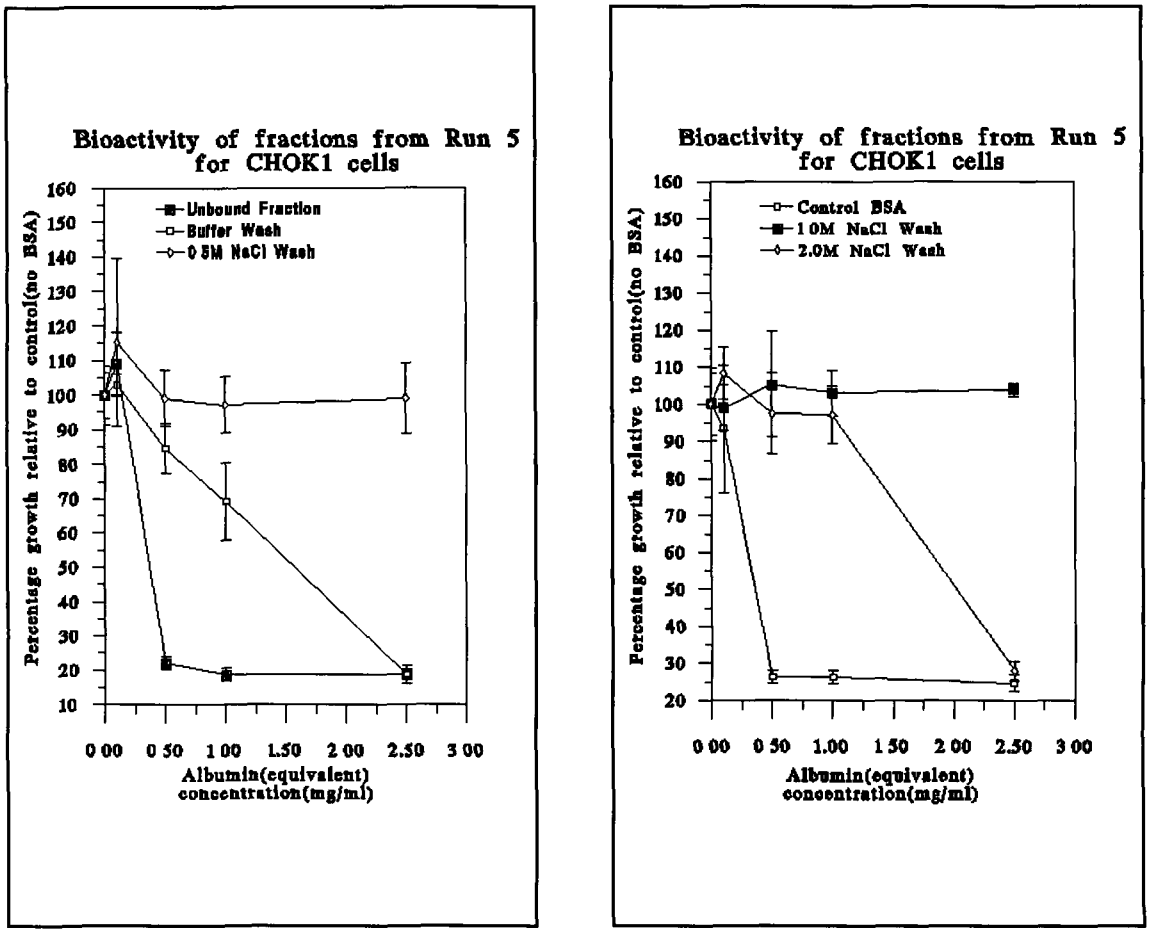


Figure 3.5.4.3.2 shows the response of CHOK1 cells to dilutions of each fraction from Run 1 (Assay 1) Results are expressed as the average growth relative to control (serum-free medium without albumin) ± standard deviation (n=8) Results were split into two graphs to allow a clearer view of each dose response curve The results for three separate assays are shown in Tables 3 5 4 3 2a - c

Table 3.5.4.3.2a Growth response of CHOK1 cells to all the fractions at albumin (equivalent) concentration

ASSAY 1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 7 01	100 0 ± 6 55	100 0 ± 6 55	100 0 ± 8 49	100 0 ± 8 49	100 0 ± 9 80
0 1 mg/ml	93 4 ± 17 1	109 0 ± 9 02	103 0 ± 3 25	115 3 ± 24 3	99 00 ± 6 07	108 2 ± 7 01
0 5 mg/ml	26 3 ± 1 61	22 00 ± 1 80	84 66 ± 7 21	99 00 ± 8 09	105 2 ± 14 2	97 40 ± 10 8
1 0 mg/ml	26 3 ± 1 61	18 77 ± 1 80	69 21 ± 11 3	97 10 ± 8 09	103 0 ± 6 07	97 12 ± 7 76
2 5 mg/ml	24 7 ± 2 13	18 77 ± 1 40	18 77 ± 2 53	99 00 ± 10 1	103 8 ± 1 65	27 93 ± 2 65

For the CHOK1 cells (Figure 3 5 4 3 5), the unbound showed 30 - 40% inhibition relative to the control (SFM no albumin) The buffer wash was only 20% inhibitory With the 0 5M NaCl fraction very little inhibition was seen until at 2 5mg/ml, when 75 - 80% inhibition occurred Little activity was seen in the 1 0 and 2 0M NaCl fractions The BSA control was inhibitory also, reaching 60% to 70% inhibition at the highest concentrations tested (5mg/ml)

It would appear that while the factor was stimulatory for the NRK cells, it was inhibitory for the CHOK1 cells

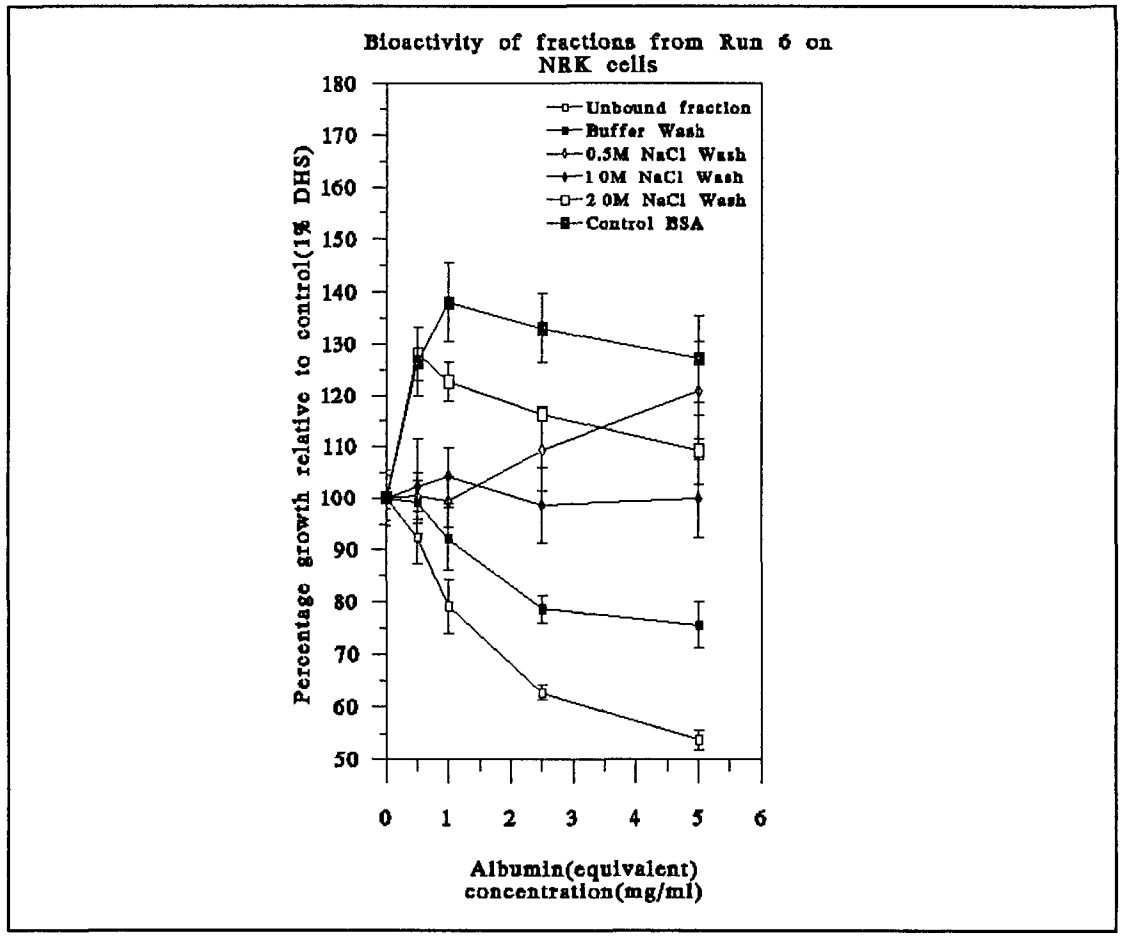


Figure 3.5.4.3.3 shows the growth response of NRK cells to a dilution curve of each fraction obtained from Run 6 on the HS column (Assay 2) Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point and results for three separate assays are shown in Tables 3 5 4 3 3a to c

Table 3.5.4.3.3a Growth response of NRK cells to all fractions at albumin (equivalent) concentration

ASSAY 1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 4 19	100 0 ± 3 66	100 0 ± 3 66	100 0 ± 4 09	100 0 ± 4 09	100 0 ± 4 19
0 5 mg/ml	125 ± 3 95	89 98 ± 8 19	81 81 ± 5 57	113 4 ± 4 38	97 41 ± 5 19	123 1 ± 6 57
1 0 mg/ml	139 ± 4 68	75 81 ± 4 83	69 79 ± 6 45	99 27 ± 4 11	95 59 ± 8 16	117 9 ± 6 47
2 5 mg/ml	141 ± 11 8	61 80 ± 4 29	72 95 ± 5 76	105 7 ± 1 77	90 23 ± 3 58	114 7 ± 9 05
5 0 mg/ml	134 ± 11 2	56 89 ± 3 51	63 91 ± 5 52	115 4 ± 6 06	90 92 ± 4 87	100 6 ± 5 38

Table 3.5.4.3 3b Growth response of NRK cells to all fractions at albumin (equivalent) concentration

ASSAY 2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 2 05	100 0 ± 5 32	100 0 ± 5 32	100 0 ± 4 43	100 0 ± 4 43	100 0 ± 2 05
0 5 mg/ml	126 ± 6 64	92 39 ± 5 04	99 23 ± 4 18	100 4 ± 4 48	102 3 ± 9 29	128 1 ± 5 23
1 0 mg/ml	138 ± 7 53	79 21 ± 5 20	92 14 ± 6 02	99 33 ± 4 82	104 3 ± 5 52	122 6 ± 3 73
2 5 mg/ml	133 ± 6 24	62 71 ± 1 33	78 66 ± 2 73	109 3 ± 7 87	98 69 ± 7 41	116 4 ± 1 37
5 0 mg/ml	127 ± 8 39	53 69 ± 1 93	75 57 ± 4 37	120 9 ± 9 43	99 93 ± 7 63	108 3 ± 6 64

Table 3.5.4 3.3c Growth response of NRK cells to all fractions at albumin (equivalent) concentration

ASSAY 3	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 5 14	100 0 ± 4 83	100 0 ± 4 83	100 0 ± 4 38	100 0 ± 4 38	100 0 ± 5 14
0 5 mg/ml	129 ± 4 53	93 21 ± 2 98	94 18 ± 6 07	96 49 ± 3 24	103 5 ± 6 54	126 9 ± 9 09
1 0 mg/ml	143 ± 6 29	76 05 ± 2 36	91 21 ± 4 99	104 8 ± 6 65	99 62 ± 5 12	123 1 ± 3 48
2 5 mg/ml	135 ± 4 09	64 73 ± 3 65	80 61 ± 2 21	113 3 ± 2 12	98 14 ± 6 26	108 1 ± 10 7
5 0 mg/ml	129 ± 4 74	55 12 ± 3 64	78 74 ± 1 57	122 4 ± 4 57	93 79 ± 5 86	103 1 ± 3 96

Table 3 5.4.3.4a Growth response of CHOK1 cells to all fractions at albumin (equivalent) concentration

ASSAY 1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 3 30	100 0 ± 3 39	100 0 ± 3 39	100 0 ± 2 18	100 0 ± 2 18	100 0 ± 3 30
0 1 mg/ml	20 7 ± 4 72	94 07 ± 5 51	86 02 ± 5 93	102 2 ± 7 40	99 47 ± 6 48	104 2 ± 4 24
0 5 mg/ml	21 2 ± 0 47	92 37 ± 4 24	91 10 ± 3 81	96 69 ± 6 48	99 47 ± 5 09	103 8 ± 10 4
1 0 mg/ml	48 3 ± 2 76	85 17 ± 5 51	88 98 ± 2 97	88 37 ± 4 63	101 3 ± 6 48	105 7 ± 8 96
2 5 mg/ml	43 4 ± 2 08	66 52 ± 4 24	67 79 ± 5 08	25 45 ± 0 92	104 1 ± 5 55	104 7 ± 7 55

Table 3 5.4.3 4b Growth response of CHOK1 cells to all fractions at albumin (equivalent) concentration

ASSAY 2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Was	2M NaCl Wash
0 0 mg/ml	100 ± 6 57	100 0 ± 4 84	100 0 ± 4 84	100 0 ± 4 12	100 0 ± 4 12	100 0 ± 6 57
0 1 mg/ml	95 7 ± 5 58	104 5 ± 3 81	102 4 ± 3 46	103 4 ± 6 18	107 6 ± 1 37	99 65 ± 3 46
0 5 mg/ml	89 9 ± 6 92	97 58 ± 7 27	103 9 ± 6 23	100 7 ± 5 15	105 8 ± 6 87	98 27 ± 5 88
1 0 mg/ml	82 7 ± 6 57	97 23 ± 5 19	107 9 ± 3 81	91 41 ± 5 15	104 8 ± 6 87	97 23 ± 4 49
2 5 mg/ml	43 9 ± 3 81	72 32 ± 3 46	82 69 ± 4 49	25 77 ± 1 37	105 8 ± 5 49	89 27 ± 5 88

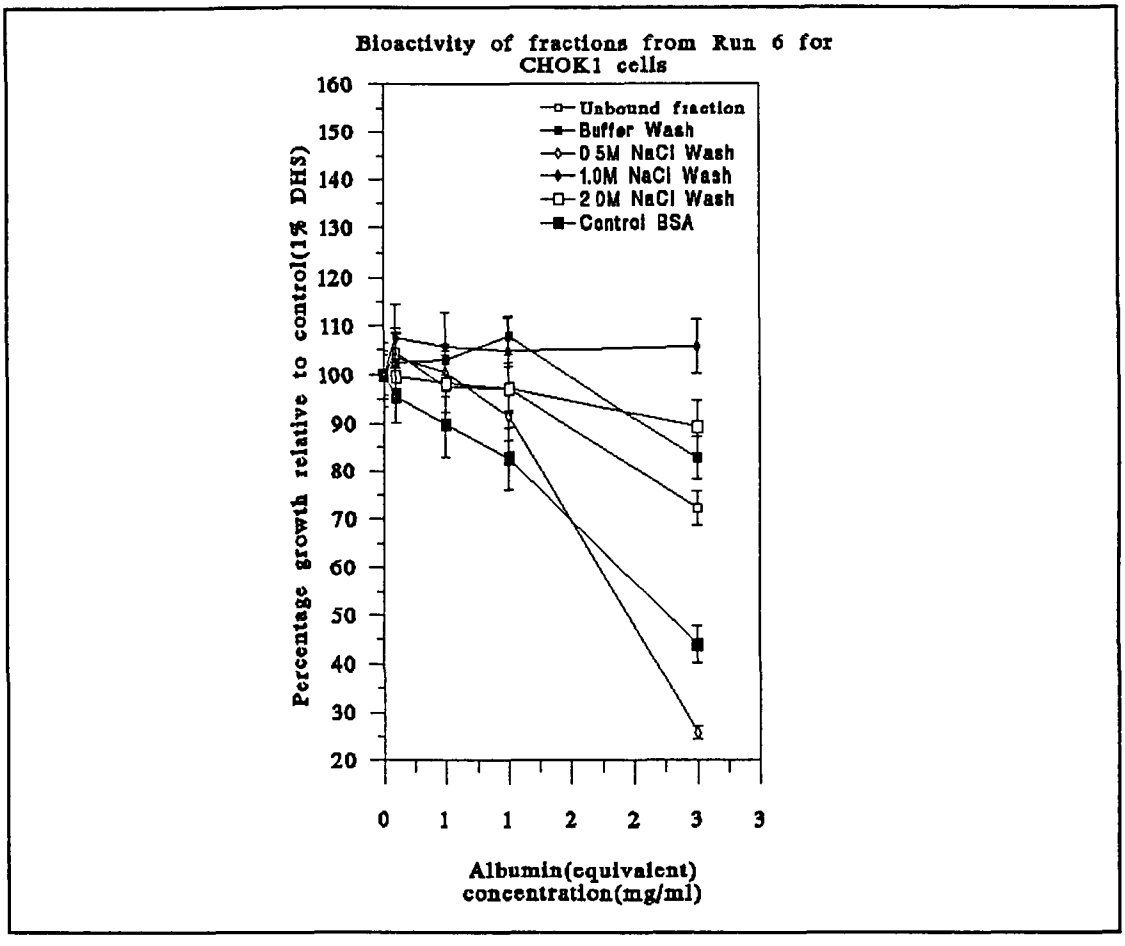


Figure 3.5.4.3.4 shows the growth response of CHOK1 cells to a dilution curve of each fraction obtained from Run 6 on the HS column (Assay 2) Results are expressed as the average percentage growth relative to control (SFM without albumin) ± standard deviation (n=8) Acid phosphatase was used as the end point and results for three separate assays are shown in Tables 3 5 4 3 4a to c

Table 3.5.4 3.4c Growth response of CHOK1 cells to all fractions at albumin (equivalent) concentration

ASSAY 3	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0 mg/ml	100 ± 3 24	100 0 ± 7 01	100 0 ± 7 01	100 0 ± 5 81	100 0 ± 5 81	100 0 ± 3 24
0 1 mg/ml	113 ± 5 31	100 9 ± 7 79	97 83 ± 6 41	98 78 ± 5 19	92 05 ± 3 67	104 4 ± 4 72
0 5 mg/ml	110 ± 3 24	112 2 ± 7 10	114 7 ± 7 36	111 9 ± 6 12	98 16 ± 3 97	97 93 ± 5 01
1 0 mg/ml	98 8 ± 3 54	104 7 ± 4 59	124 7 ± 6 84	107 6 ± 2 45	115 3 ± 1 80	104 4 ± 5 31
2 5 mg/ml	33 3 ± 1 77	61 73 ± 4 50	99 57 ± 3 55	21 71 ± 1 53	109 2 ± 3 97	100 6 ± 3 24

3.5.4.4 Variable response of different end points to HS fractions: Effect of assay system

In Run 6, the maximum stimulation of the 0 5M NaCl fraction and the BSA control for NRK cells were 20% and 40% stimulation above the control (1% DHS) at 5 0mg/ml and 2 5mg/ml respectively When the fractions were assayed on 24-well plates, using image analysis and dye elution as end points, stronger stimulation in the 0 5M NaCl fraction was observed (Figure 3 5 4 4 1)

Image analysis showed a 2 7-fold and 3 1-fold increase in growth over the control for the 0 5M NaCl wash and the control BSA respectively When the crystal violet dye was eluted off the plates and the absorption measured, about 70% stimulation over the control was observed for both 0 5M NaCl and control BSA Acid phosphatase (from 96-well plates) showed only 20 and 30% stimulation over the control respectively Image analysis results were much higher For image analysis, measurement is based on colony area and so results may reflect cell spreading in addition to cell number

To investigate whether acid phosphatase (AP) or dye elution (DE) was most representative of cell number, a dilution curve of the BSA was assayed in 24-well plates Results were analyzed using AP, cell number and dye elution as the end points The results are shown in Figure 3 5 4 4 2

Although there was variability in the three repeat experiments, all three end points showed similar results This would indicate that acid phosphatase was almost as good as dye elution in 24-well plates However in 96-well plates, as seen above, used of DE resulted in higher growth

The effect observed in the 96-well plates may have been due to limited growth stimulation as a result of an initial high inoculation density or to a failure of AP levels to rise in proportion of cell number To see if the inoculation density was affecting the observed activity for AP in 96-well plates, four different initial cell densities were set up in duplicate, to assay the effects of the control BSA The results are seen in Figures 3 5 4 4 3 and 3 5 4 4 4 One set of plates were taken down using AP as the end point and the second were taken down using DE as the end-point In both cases the higher initial cell density gave marginally better stimulation.

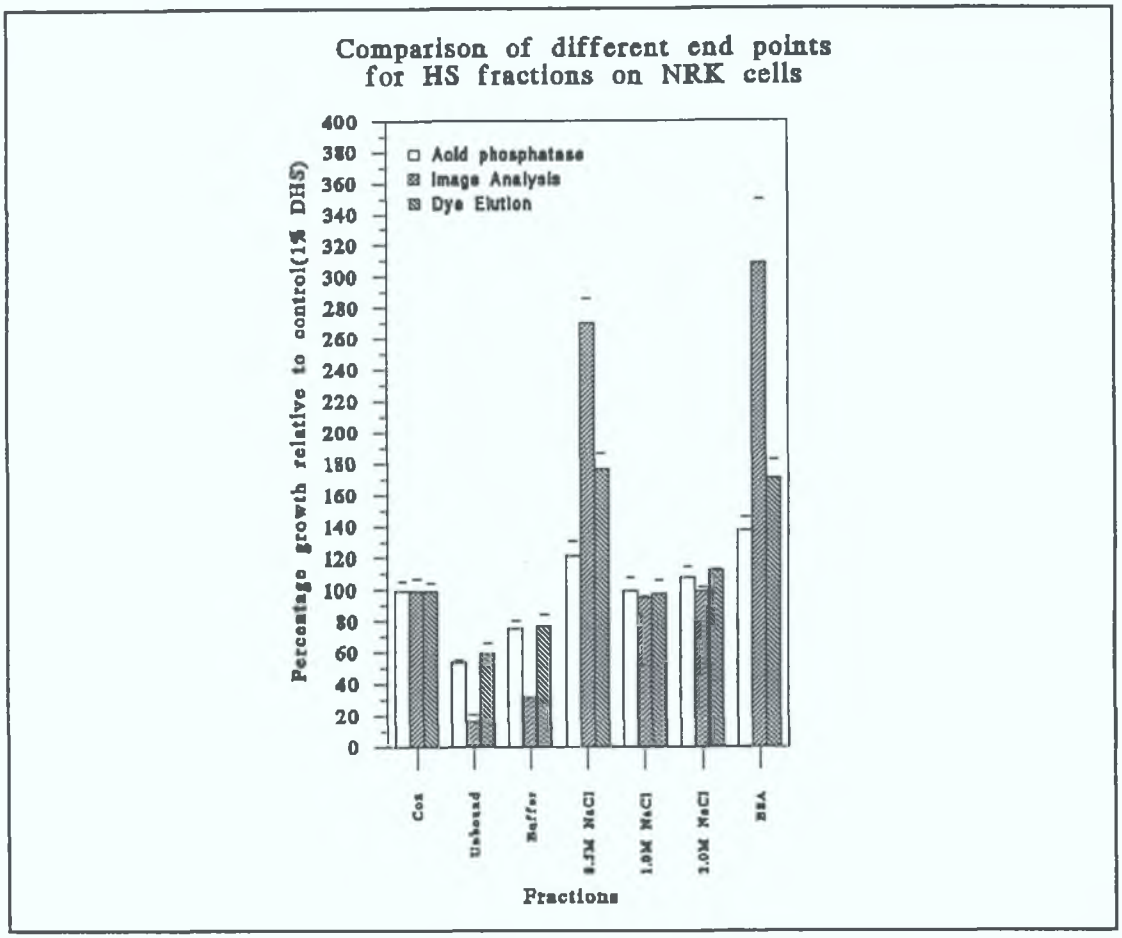


Figure 3.5.4.4.1 shows the average growth response of NRK cells to the heparin sepharose fractions and the BSA at 5mg/ml albumin concentration. Three end points were used. Cells set up in 96-well plates were read using acid phosphatase (AP) as the end point. Cells set up in 24-well plates were stained with crystal violet and the colony area was measured using image analysis (IA). The dye was then eluted off the plate and the absorbance measured at a dual wavelength (570 and 620nm). This was referred to as dye elution (DE). Results are expressed as the average percentage growth relative to control (1% DHS) $\pm$ standard deviation (n=8).

Table 3.5.4.4.1 Effect of different end points on the ability of BSA to stimulate NRK cells

End Point	BSA	Unbound fraction	Buffer Wash	0.5M NaCl Wash	1.0M NaCl Wash	2.0M NaCl Wash
AP	133.5 $\pm$ 11.2	56.89 $\pm$ 3.51	63.91 $\pm$ 5.52	115.4 $\pm$ 7.65	90.92 $\pm$ 4.86	100.6 $\pm$ 5.38
	137.1 $\pm$ 8.40	53.69 $\pm$ 1.93	75.51 $\pm$ 4.37	120.9 $\pm$ 6.14	99.93 $\pm$ 7.63	108.3 $\pm$ 6.64
	127.0 $\pm$ 4.74	55.12 $\pm$ 3.64	78.74 $\pm$ 1.57	122.4 $\pm$ 11.0	93.79 $\pm$ 5.86	103.2 $\pm$ 3.96
IA	100.0 $\pm$ 12.5	17.00 $\pm$ 3.69	31.29 $\pm$ 0.50	270.5 $\pm$ 7.89	94.90 $\pm$ 15.9	99.77 $\pm$ 2.14
	301.8 $\pm$ 11.8	15.61 $\pm$ 1.17	133.6 $\pm$ 40.1	439.2 $\pm$ 47.4	130.2 $\pm$ 42.4	300.8 $\pm$ 3.90
DE	100.0 $\pm$ 22.7	70.41 $\pm$ 6.71	120.9 $\pm$ 13.7	211.9 $\pm$ 12.9	107.3 $\pm$ 7.12	150.2 $\pm$ 6.23
	307.7 $\pm$ 57.1	59.40 $\pm$ 6.77	76.77 $\pm$ 7.24	176.9 $\pm$ 9.68	97.87 $\pm$ 8.52	111.8 $\pm$ 0.89

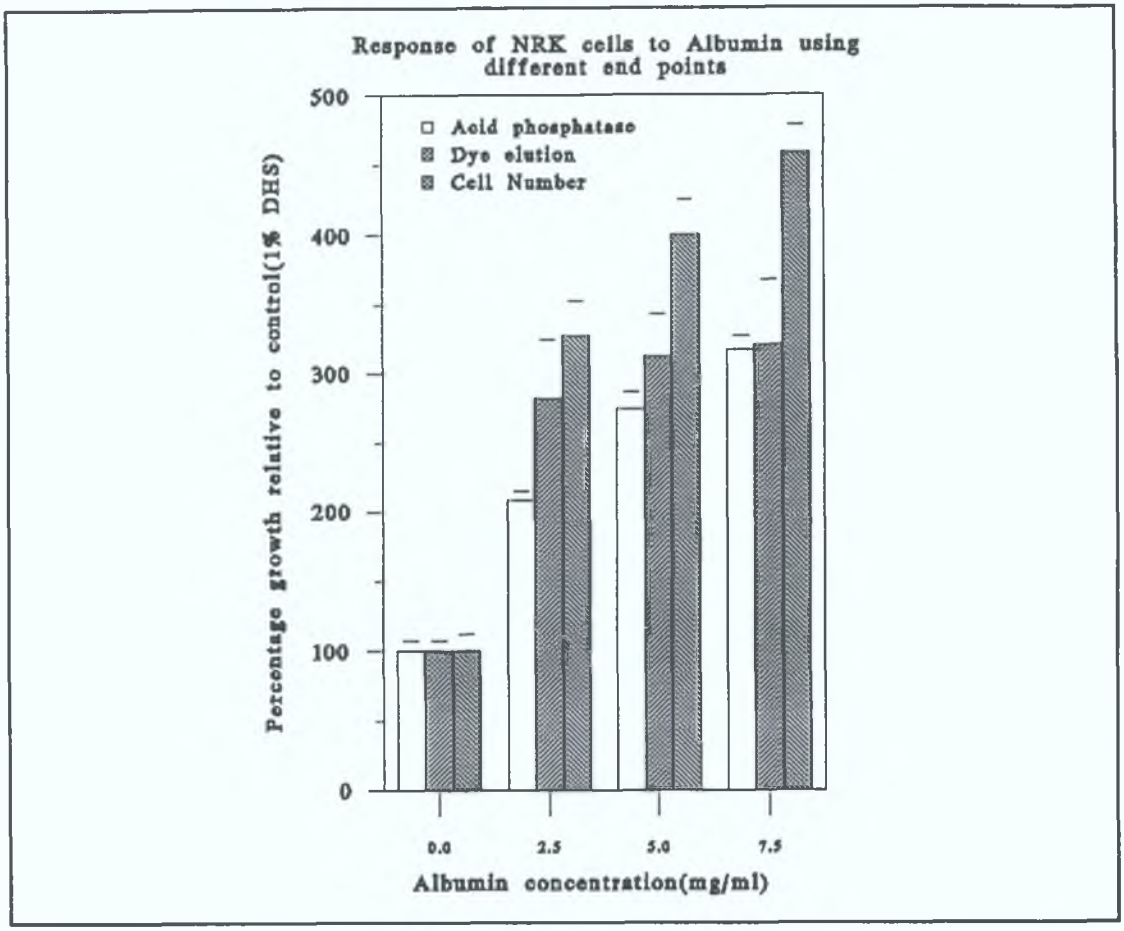


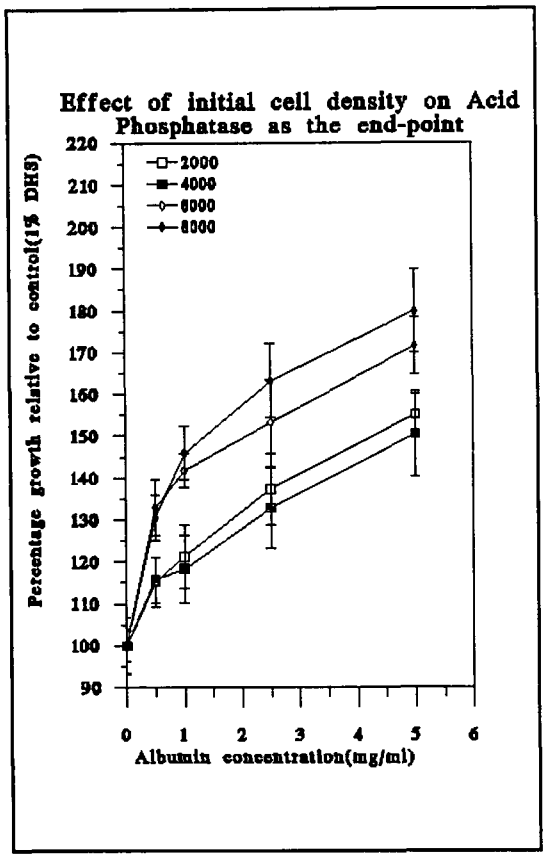
Figure 3.5.4.4.2 shows the average growth response of NRK cells to mg/ml albumin concentrations using different end points (Assay 2). Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8). Abbreviations: AP = acid phosphatase; DE = dye elution; CN = cell number.

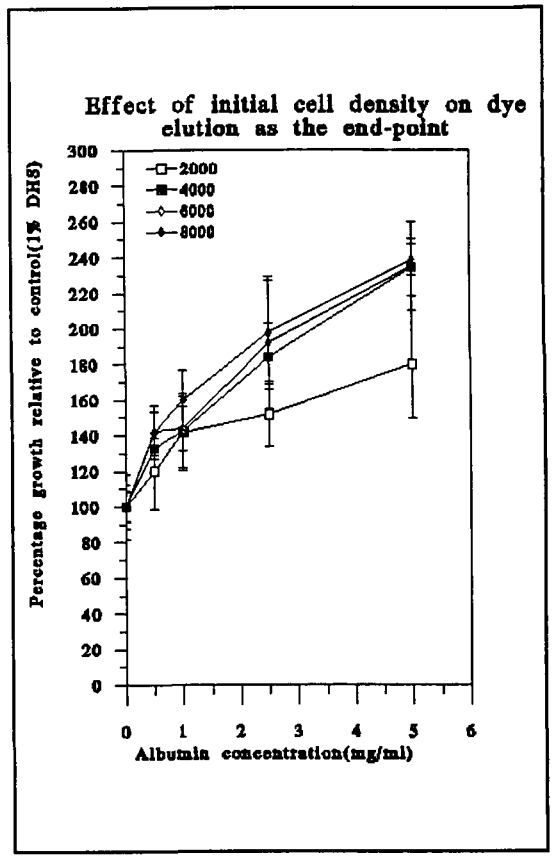
Table 3.5.4.4.2 Effect of different end points on the ability of BSA to stimulate NRK cells (mg/ml)

END POINT	ASSAY 1	ASSAY 2	ASSAY 3
AP: 0.0mg/ml	100.0 ± 7.26	100.0 ± 7.65	100.0 ± 6.58
2.5mg/ml	204.2 ± 41.9	208.6 ± 6.14	194.0 ± 24.1
5.0mg/ml	250.3 ± 24.4	274.0 ± 12.5	238.2 ± 17.9
7.5mg/ml	268.4 ± 39.3	316.5 ± 11.0	330.4 ± 3.45
DE: 0.0mg/ml	100.0 ± 12.5	100.0 ± 7.89	100.0 ± 5.80
2.5mg/ml	288.5 ± 31.4	281.2 ± 42.4	258.4 ± 71.9
5.0mg/ml	303.3 ± 7.75	312.1 ± 31.5	346.1 ± 9.52
7.5mg/ml	301.8 ± 11.8	321.1 ± 47.4	372.2 ± 61.5
CN: 0.0mg/ml	100.0 ± 22.7	100.0 ± 12.9	100.0 ± 23.6
2.5mg/ml	246.6 ± 62.4	327.0 ± 25.7	394.4 ± 25.5
5.0mg/ml	280.3 ± 21.3	400.0 ± 25.7	441.7 ± 11.8
7.5mg/ml	307.7 ± 57.1	459.1 ± 19.3	491.7 ± 35.3

However, the extent of stimulation with DE was approximately twice that observed with AP Thus the initial cell density did not account for the differences in results obtained with the two end points, as lower initial cell densities did not result in better stimulation

Investigation into the end point used for the detection of bioactivity showed that while in 24-well plates, acid phosphatase, dye elution and cell number all showed good stimulation, the extent of stimulation seen with acid phosphatase in 96-well plates was much lower Crystal violet dye elution from 96-well plates showed similar results to those obtained for cell counts in 24-well plates The possibility that the initial seeding density of NRK cells was too high was also investigated (Figures 3 5 4 4 3 and 3 5 4 4 4) The higher cell density showed better growth than the lower cell densities tested However, cells were normally plated at $8x10^3$ cells/ml Why acid phosphatase appeared to give greater stimulation relative to the control (1% DHS) in 24-well than in 96-well plates is unknown As crystal violet dye elution from 96-well plates showed the same trend as that obtained by cell number, dye elution from 96-well plates was chosen as the end point for future assays





Figures 3.5.4.4.3 and 3.5.4.4.4 show the effect of the initial cell density on acid phosphatase and dye elution as end points when looking at the response of NRK cells to albumin. Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) The results for three separate assays are shown in Tables 3 5 4 4 3 and 3 5 4 4 4a,b and c

Table 3.5.4.4.3a Effect of initial cell density (Cells/ml) on BSA stimulated activity (mg/ml)

ASSAY 1	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 3 75	100 0 ± 3 81	100 0 ± 4 36	100 0 ± 2 95
0 5 mg/ml	120 5 ± 8 66	121 9 ± 3 14	120 7 ± 3 79	124 7 ± 3 30
1 0 mg/ml	125 8 ± 6 29	128 2 ± 7 82	132 9 ± 8 02	146 1 ± 4 78
2 5 mg/ml	140 1 ± 5 50	149 2 ± 7 33	149 8 ± 6 75	161 2 ± 6 18
5 0 mg/ml	156 7 ± 10 2	154 4 ± 8 90	165 4 ± 13 9	181 2 ± 5 98

Table 3.5.4.4.3b Effect of initial cell density (Cells/ml) on BSA stimulated activity (mg/ml)

ASSAY 2	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 5 31	100 0 ± 4 21	100 0 ± 5 12	100 0 ± 3 73
0 5 mg/ml	111 3 ± 9 02	123 9 ± 8 33	122 7 ± 10 5	124 9 ± 5 54
1 0 mg/ml	116 5 ± 6 01	126 7 ± 6 25	130 8 ± 10 9	135 4 ± 5 54
2 5 mg/ml	122 7 ± 5 72	147 9 ± 5 21	152 2 ± 10 9	152 6 ± 6 77
5 0 mg/ml	133 8 ± 5 26	171 4 ± 6 63	177 9 ± 9 92	172 6 ± 7 69

Table 3.5.4.4.3c Effect of initial cell (cells/ml) density on BSA stimulated activity (mg/ml)

ASSAY 3	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 6 86	100 0 ± 6 71	100 0 ± 3 86	100 0 ± 3 67
0 5 mg/ml	115 2 ± 5 93	115 6 ± 5 38	132 9 ± 6 76	130 5 ± 5 50
1 0 mg/ml	121 2 ± 7 63	118 3 ± 8 06	141 9 ± 4 05	145 9 ± 6 25
2 5 mg/ml	137 3 ± 8 47	132 8 ± 9 68	153 1 ± 10 4	163 2 ± 8 82
5 0 mg/ml	155 1 ± 5 08	150 5 ± 10 2	171 6 ± 6 76	179 8 ± 9 93

Table 3.5.4.4.4a Effect of initial cell density (cells/ml) on BSA stimulated activity (mg/ml)

ASSAY 1	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 12 1	100 0 ± 18 3	100 0 ± 8 22	100 0 ± 8 69
0 5 mg/ml	120 0 ± 21 4	132 9 ± 5 51	142 3 ± 14 6	141 2 ± 11 8
1 0 mg/ml	141 7 ± 20 0	142 2 ± 21 5	144 0 ± 12 4	159 7 ± 8 61
2 5 mg/ml	151 7 ± 18 1	183 9 ± 18 6	191 7 ± 37 1	197 5 ± 16 9
5 0 mg/ml	180 0 ± 30 0	234 1 ± 16 0	235 0 ± 24 7	238 6 ± 29 2

Table 3.5.4.4.4b Effect of initial cell density (cells/ml) on BSA stimulated activity (mg/ml)

ASSAY 2	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 15 7	100 0 ± 3 52	100 0 ± 8 89	100 0 ± 9 64
0 5 mg/ml	125 0 ± 16 7	139 3 ± 28 9	153 8 ± 15 9	153 4 ± 13 5
1 0 mg/ml	125 0 ± 16 7	146 1 ± 16 2	177 6 ± 19 2	175 9 ± 13 5
2 5 mg/ml	141 7 ± 16 7	209 4 ± 31 4	217 4 ± 25 9	230 1 ± 27 7
5 0 mg/ml	200 0 ± 25 0	234 7 ± 20 4	272 7 ± 2 72	270 7 ± 13 5

Table 3.5.4.4.4c Effect of initial cell density (cells/ml) on BSA stimulated activity (mg/ml)

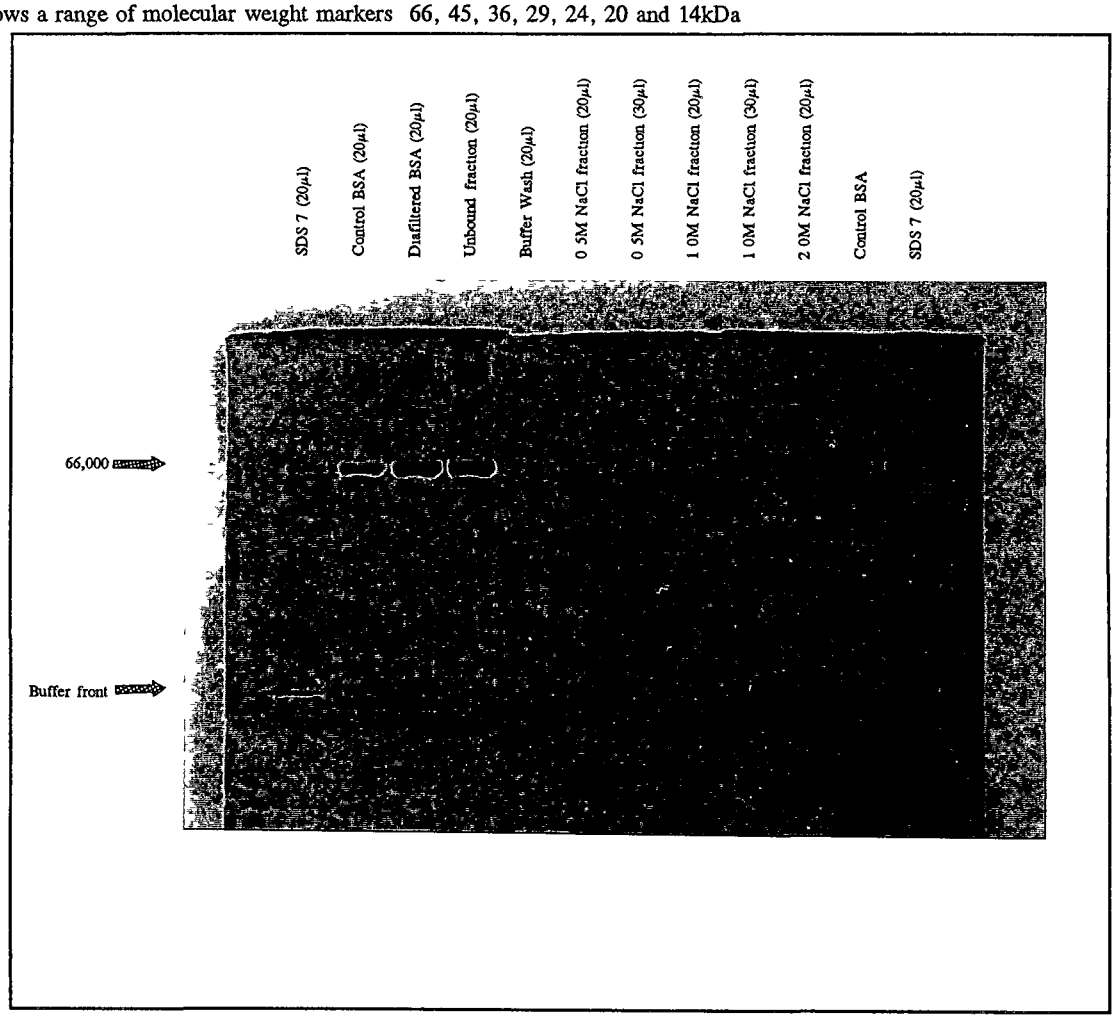
ASSAY 3	$2x10^4$	$4x10^4$	$6x10^4$	$8x10^4$
0 0 mg/ml	100 0 ± 11 4	100 0 ± 7 32	100 0 ± 7 88	100 0 ± 11 8
0 5 mg/ml	122 7 ± 21 6	117 9 ± 10 7	225 0 ± 20 4	141 4 ± 10 3
1 0 mg/ml	137 1 ± 11 8	133 9 ± 26 7	262 9 ± 22 6	158 6 ± 10 3
2 5 mg/ml	170 8 ± 13 4	182 1 ± 21 4	231 8 ± 13 6	186 2 ± 16 9
5 0 mg/ml	164 8 ± 14 0	219 6 ± 21 4	320 4 ± 34 1	213 8 ± 6 89

3.5.4.5 SDS Gels of Fractions from Heparin Sepharose

Now that activity was consistently being isolated from the bulk of the protein, various methods were used to determine what was causing the activity A more sensitive method of protein detection was used by applying samples from HS fractions (**Run 4**) to an SDS - PAGE gel and staining with silver stain

Gels were prepared according to Laemmli and Favre (1973) and stained with silver stain (Oakley *et al* 1980) The gel showed that the protein present in the 0 5M NaCl wash had the same molecular mass as the major band of the albumin control No smaller molecular mass bands were observed in the 0 5M NaCl fraction

Figure 3.5.4.4 1 SDS gel of fractions from HS fractionation of BSA Varying volumes of 20 to 30µl were applied to gels with a concentration of 10ng/ml (see materials and methods, section 2 13 3) The SDS control shows a range of molecular weight markers 66, 45, 36, 29, 24, 20 and 14kDa



It may be that the protein in the 0 5M NaCl fraction is a subpopulation of the BSA or that some bound factor imparts the activity to the albumin The albumin may also be present in the 0 5M NaCl fraction due to insufficient washing of the column The 1 0M and 2 0M NaCl fractions showed no detectable protein in either fraction

3.5.4.6 Trypsin Activity on HS Fractions

In two of the four previous experiments, the biological activity of albumin was isolated in the 0 5M NaCl fraction In an attempt to ascertain whether or not the activity was due to the small amount of protein sometimes measured in the 0 5M NaCl wash, it was decided to subject the fraction to trypsin digestion The 0 5M NaCl fraction from RUN 4 was used The resulting digest was assayed for bioactivity on NRK cells as before The digest was also run on an SDS-PAGE gel and stained with silver stain (as in 3 5 4 5)

Trypsin digestion was carried out as described in Section 2 18 Briefly, the samples were exposed to trypsin, allowed to incubate for 2hrs at 37°C Trypsin inhibitor was then added The results for two separate experiments are shown in Tables 3 5 4 6 1 and 2
In the first experiment (Table 3 5 4 6 1), growth stimulation seen with the BSA and 0 5M NaCl fraction was very low, making trends difficult to observe The low activity was most likely due to the use of acid phosphatase as the end point

Table 3.5.4.10.1 Effect of trypsin digestion on HS fractions

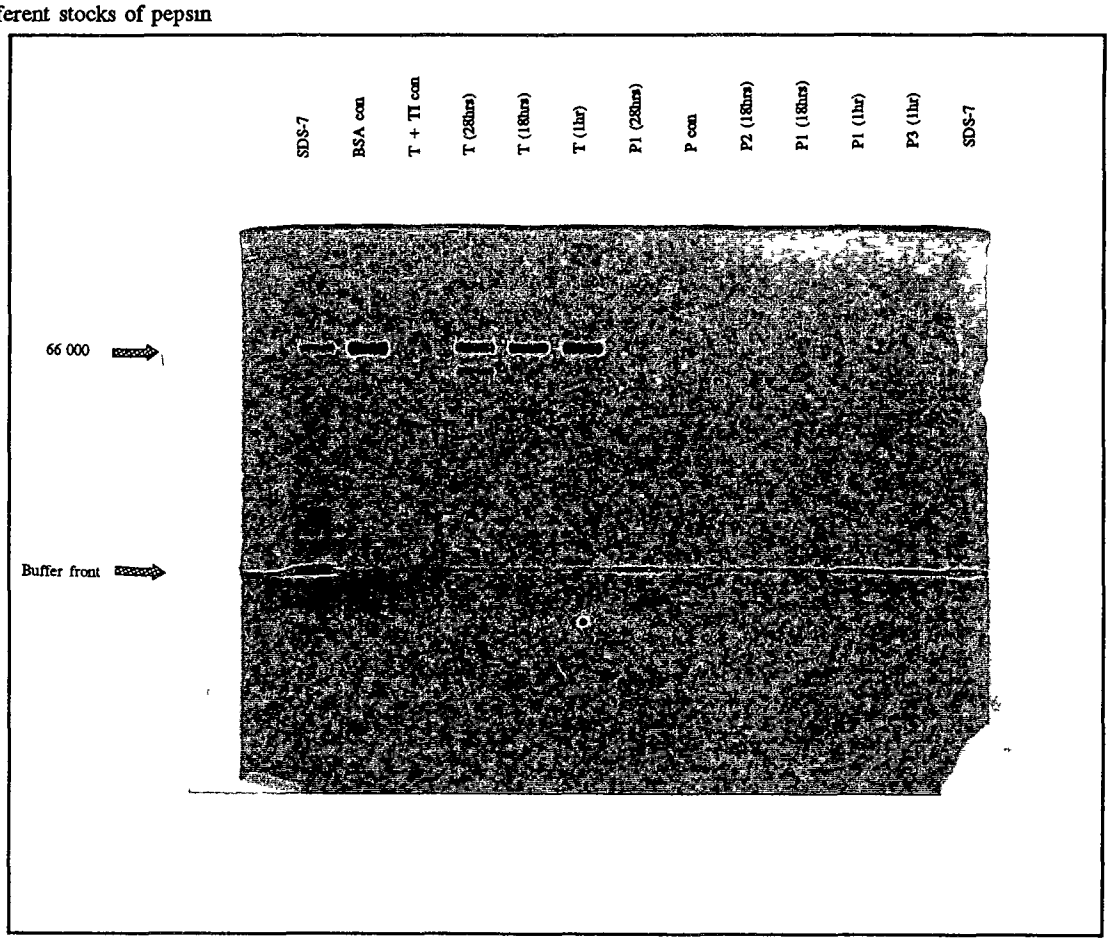
VARIABLES	SAMPLE	A	B	C
ATCC	100 0 ± 3 0	97 6 ± 3 6	101 2 ± 1 8	95 5 ± 1 8
0 5M NaCl	127 0 ± 2 4	143 3 ± 7 3	46 3 ± 7 3	82 9 ± 1 2
0 5M NaCl*	90 2 ± 10 4	90 8 ± 3 0	87 2 ± 3 0	87 8 ± 2 4
Control BSA	109 9 ± 6 4	119 9 ± 7 4	107 4 ± 2 5	45 9 ± 0 6
New BSA	129 2 ± 5 6	132 3 ± 3 7	130 4 ± 4 9	125 5 ± 4 9

Results show the effect of trypsin digestion on the heparin sepharose fractions from run 4 0 5M NaCl* refers to the 0 5M fraction obtained when the column was run without albumin Growth is expressed as the average percentage growth relative to the control (ATCC + 1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point Abbreviations A = sample plus trypsin inhibitor, B = sample plus trypsin, incubated for 2hrs before addition of trypsin inhibitor, C = sample plus trypsin inhibitor, followed by addition of trypsin, New BSA = fresh stock of BSA

To see how effective trypsin and pepsin were at degrading BSA, samples were exposed to each and the resulting digests were run on an SDS gel The results are shown in the gel in Table 3 5 4 6 1 Trypsin after 1 hour, had not caused any change in the migration position of the albumin However, after 18hrs, a second smaller band appeared The second band was even more distinct at 24hrs Pepsin was much more effective After only one hour, no protein was detectable at the position to which albumin normally migrated No other distinct bands were visible

These results indicated that trypsin did have an effect on albumin over extended incubation times As Pepsin was too harsh, it was hoped that the shorter times of 1 to 2hrs with trypsin should affect any protein that the albumin was carrying

Figure 3.5.4.6.1 Gel showing trypsin and pepsin digests Samples were applied at 30μl per well with a concentration of 10ng/ml (see materials and methods 2 13 3) The SDS control shows a range of molecular weight markers 66, 45, 36, 29, 24, 20 and 14kDa Abbreviations T = trypsin, TI = trypsin inhibitor, P1, 2 and 3 = three different stocks of pepsin



In a second experiment, samples from **Run 7** were exposed to trypsm digestion The results from this experiment showed that trypsin appeared to have some effect on the 0 5M NaCl fraction, but not on the BSA control or on the ATCC control (Table 3 5 4 6 2)

Addition of trypsin resulted in a loss of stimulation 30 6%, 54 5% and 40 5% stimulation relative to the undigested 0 5M NaCl fraction for assays 1, 2 and 3 respectively relative to the control 0 5M NaCl If the trypsm inhibitor control were used as a basis of determining loss of activity, 23 3, 43 8 and 39 7% of the activity remained after treatment with trypsin for the three separate assays respectively The results for the 0 5M NaCl were complicated for two

reasons When the trypsin inhibitor was added before the trypsin, a decrease in activity of 20 - 30% was still seen This decrease was not seen in the BSA and ATCC controls This could indicate that the 0 5M NaCl fraction sufficient trypsin inhibitor was not present to inactivate the trypsin immediately or that some of the active component in the 0 5M NaCl fraction was susceptible to trypsin However, the trypsin was apparently inactivated by the trypsin inhibitor as seen in the ATCC controls The fact that there was a lower amount of protein in the 0 5M NaCl fraction may have made the active fraction more susceptible to the action of trypsin than the BSA

In addition, the effect of the trypsm inhibitor again appeared to cause an increase in growth response This increase was variable between the three assays, up to 30% For ATCC, the stimulation was variable also (no greater than 30%) For the BSA, stimulation doubled over the BSA on its own (2 4-, 2 56- and 3 68-fold increase for the three assays respectively)

Table 3.5.4.6.2. Effect of trypsin digestion on bioactivity of HS fractions from run 7

VARIABLES	SAMPLE	A	B	C
Assay 1 BSA	158 3 ± 16 0	240 0 ± 16 3	157 0 ± 18 6	168 0 ± 16 0
0 5M NaCl	246 6 ± 17 0	293 0 ± 27 0	145 0 ± 13 8	190 0 ± 18 9
ATCC	93 6 ± 10 6	114 0 ± 0 0	101 5 ± 9 9	96 50 ± 10 7
Assay 2 BSA	194 1 ± 30 4	341 2 ± 23 5	174 5 ± 25 1	243 1 ± 28 5
0 5M NaCl	297 1 ± 33 8	350 0 ± 36 4	209 4 ± 21 0	230 6 ± 19 7
ATCC	74 6 ± 7 46	80 60 ± 6 2	74 6 ± 8 2	80 60 ± 8 2
Assay 3 BSA	165 0 ± 12 5	339 3 ± 41 1	173 2 ± 15 2	179 2 ± 18 8
0 5M NaCl	353 1 ± 27 7	358 3 ± 27 0	202 5 ± 10 5	245 6 ± 17 1
ATCC	96 60 ± 8 2	130 0 ± 12 2	101 6 ± 4 1	100 0 ± 6 3

Results from three separate assays are shown Growth is expressed as the average percentage growth relative the control (ATCC + 1% DHS) ± standard deviation (n=8) Dye elution was used as the end point Abbreviations A = sample plus trypsin inhibitor, B = sample plus trypsin, incubated for 2hrs before addition of trypsin inhibitor, C = sample plus trypsm inhibitor, followed by addition of trypsin

In conclusion for both experiments, a decrease in activity was seen with the 0 5M NaCl fraction However, addition of trypsin inhibitor interferred with results (the trypsm inhibitor was stimulatory to NRK cells both in the presence and absence of BSA or the 0 5M NaCl fraction) To overcome this problem, it was decided to use pepsin as a means of digestion

3.5.4.7 Pepsin Treatment of BSA and 0.5M NaCl Fraction

Samples of the BSA and 0 5M NaCl fraction were exposed to pepsin (2% wt/vol of the protein concentration) after the pH was adjusted to 2 6 The samples were incubated for 1 hour at 37°C The pH was re-adjusted to 7 4 The samples were filter sterilized and assayed for growth stimulation The experiment was carried out with BSA at an earlier stage where acid phosphatase was used as an end point, which may explain the low stimulation obtained with the BSA control Dye elution was used as the end point for the experiment with the 0 5M NaCl fraction The results are shown in Table 3 5 4 7

The results showed that the lowering of the pH caused a reduction in activity of the 0 5M NaCl, but no reduction in the native BSA Pepsin caused only a slight further decrease in the activity of the 0 5M NaCl fraction The pepsin control in which ATCC was subjected to pepsin treatment, showed no variation from the growth seen with 1% DHS only

Table 3.5.4.7 Effect of pepsin digestion on the activity of BSA and the 0 5M NaCl fraction

SAMPLE	ASSAY 1	ASSAY 2	ASSAY 3
BSA	162 4 ± 3 76	170 3 ± 9 19	168 9 ± 10 5
BSA at pH2 6	158 6 ± 5 38	157 8 ± 5 94	151 1 ± 5 00
BSA + Pep	145 7 ± 8 06	160 5 ± 9 19	156 7 ± 8 33
ATCC + Pep	105 9 ± 7 79	106 5 ± 7 03	107 2 ± 6 66
0 5M NaCl	194 0 ± 13 9	204 7 ± 29 5	215 8 ± 23 3
0 5M NaCl at pH2 6	114 8 ± 9 86	136 1 ± 13 7	169 2 ± 17 5
0 5M NaCl + Pep	110 1 ± 13 1	112 6 ± 25 4	126 0 ± 8 84

Results show the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point for three separate assays for BSA while the 0 5M NaCl samples were carried out using dye elution as the end point The 'BSA and 0 5M NaCl at Ph 2 6' refers to a control in which each sample was exposed to pH2 6 as if pepsin were present After a 1hr incubation, the pH was returned to 7 4

The results showed that 20% of the activity associated with the BSA was lost after pepsin digestion relative to the BSA control (even though the pepsin appeared to digest fully the BSA on the SDS gel) For the 0 5M NaCl fraction, only 10 - 22% of the activity seen in the 0 5M NaCl control was left after digestion The results also suggested that the 0 5M NaCl fraction unlike the BSA may have been acid labile If this was the case, then the loss of stimulation seen with pepsin digestion may be due to pH

Pepsin digestion was repeated at a later stage The results are shown in Table 3 5 4 7a The pH again affected growth with a 40 - 50% loss in activity relative to the 0 5M NaCl control with little or no further loss in stimulation at pH2 6 on addition of pepsin Pepsin alone at neutral pH had variable effect on the stimulation of the 0 5M NaCl fraction

Table 3.5.4.7a

VARIABLES	ASSAY 1		ASSAY 2	
	Growth	% ret	Growth	% ret
1% DHS	100 0 ± 6 60	--	100 0 ± 9 89	--
0 5M NaCl fraction	275 8 ± 17 2	--	185 3 ± 20 9	--
0 5M + Pep	224 1 ± 17 2	70 5	185 3 ± 10 9	100 0
0 5M at pH2 6	189 6 ± 17 2	50 9	152 9 ± 15 6	62 00
0 5M + Pep at pH2 6	193 1 ± 20 7	52 9	156 5 ± 9 95	66 24

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations % ret = percentage of activity retained after treatment relative to the 0 5M NaCl fraction Pep = pepsin at 2% weight/volume final concentration

3.5.4.8 Osmolarity of HS Fractions

As elution of the activity occurred with increasing salt concentration, the possibility that increases in the osmolarity were responsible for activity were investigated Fractions from runs 2 to 8 were tested using an osmometer Dilutions of each fraction were made so that albumin concentration was at 5mg/ml (the highest concentration tested on the cells)

Table 3.5 4.8 Osmolarity for HS fractions (Osmols/Kg)

RUN	BSA	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl WASH	1 0M NaCl WASH	2 0M NaCl WASH
1	0 331	0 315	0 300	**0 327**	0 349	---
2	0 348	0 322	0 321	**0 362**	0 339	0 351
3	0 314	0 313	0 311	0 328	**0 319**	0 330
4	0 284	0 279	0 290	**0 292**	0 293	0 282
5	0 315	0 299	0 299	**0 313**	0 316	0 317
6		0 303	0 307	**0 341**	0 325	0 318
7	0 328	0 299	0 292	---	0 316	0 315
8	0 324	0 294	0 294	**0 313**	0 298	0 312

The embolded figures show the fractions which gave maximal stimulation The underlined figures show the fractions which had the highest osmolarity readings Excluding run 7(as no sample was remaining to test the osmolarity), 3 of the 7 runs showed maximal activity to correspond with highest osmolarity If high osmolarity was responsible for the activity, then increasing the osmolarity would have resulted m increased activity However, some of the fractions showed high osmolarity but little or no stimulation, for example the 2 0M NaCl wash in run 2 had a maximum of 10% stimulation above the control while the osmolarity was quite high In addition, the differences in osmolarity readings were so small, that it was unlikely to affect cell growth to any large extent

3.5.4.9 Determination of the (non-esterified) fatty acid content of HS fractions

Fatty acids have been most commonly cited as the source of the biological activity associated with albumin To investigate the possibility that the activity of the 0 5M NaCl fractions was due to fatty acids, a biochemical determination kit was obtained from Boehringer Mannheim (Cat No 1 383 175) This kit was designed to determine non-esterified fatty acids in serum or plasma The results are shown in Table 3 5 4 13 for Run 7 Samples were left in a concentrated form in order to have the best chance of obtaining values in the linear part of the assay system (0 0 - 1 5mM)

Table 3 5 4.9 Fatty acid for HS fractions (mM)

RUN	BSA	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl WASH	1 0M NaCl WASH	2 0M NaCl WASH
FFA	0 131	0 044	0 011	0 018	0 011	0 002
BA	205 3	103 0	123 0	321 0	91 90	109 0

Abbreviations FFA = free fatty acids, BA = Biological activity, shown as the average percentage growth relative to control (1% DHS)

The linearity of this colorimetric assay is 1 5mM The standard was supposed to be 0 35mM, but a value of 0 266mM was obtained for the standard for 3 separate solutions The low readings for the standard and the fractions would indicate that a more sensitive method was required However, when the BSA control and the 0 5M NaCl fractions were compared, a 7-fold decrease in free fatty acid concentration was found The majority of fatty acids were found in the unbound fraction It also appeared that some fatty acids may have been lost during diafiltration These results taken together with the extraction experiments would indicate that the activity associated with the 0 5M NaCl fraction was not due to non-esterified fatty acids

3.5.4.10 Determination of the citric acid content of HS fractions

Citrate has been mentioned as a contaminant of albumins from plasma purifications (Hanson and Ballard, 1968) Citrate was shown to be the stimulatory fraction of BSA for rabbit pre-implantation blastocytes (Kane, 1990) In order to investigate the possibility that citrate was responsible for the activity, samples from HS fractions before diafiltration against ATCC were assayed for citrate

A kit for the determination of citrate was supplied by Boehringer Mannheim (Cat No 139076) The method was based on the conversion of citric acid (citrate) to oxaloacetate and acetate in the presence of the enzyme, citrate lyase The oxaloactete and NADH were the substrates for the second reaction catalysed by malate dehydrogenase and led to the formation of malate and NAD^+ The amount of NAD^+ produced was stoichiometric to the amount of citrate present and was measured by absorbance at 340nm The results are shown below in Table 3 5 4 14

Table 3.5.4 10.1 Citrate concentration in HS fractions from RUN 4

VARIABLES	CITRATE (mg/ml)	BIOACTIVITY (%)
BSA control	0 001	163 8 $\pm$ 13 3
Unbound Fraction	0 005	94 66 $\pm$ 12 62
Buffer Wash	0 005	105 5 $\pm$ 5 67
0 5M NaCl Wash	0 005	169 3 $\pm$ 2 53
1 0M NaCl Wash	0 005	113 1 $\pm$ 7 28
2 0M NaCl Wash	0 000	108 7 $\pm$ 10 20
NaPi Buffer	0 005	--------
Citrate standard	0 454	--------

Results show the concentrations of citrate present in HS fractions before diafiltration Bioactivity is given as the average percentage growth relative to control (1% DHS) $\pm$ standard deviation (n=8) as determined with growth of NRK cells

The results indicated that very little citrate was present in any of the samples Indeed, more citrate appeared in the fractions eluting off the column than was in the BSA control Growth stimulation did not coincide with increased citrate levels A low level of citrate was present in the Na_2HPO_4 buffer, and this may have been responsible for the presence of citrate in all fractions, except the BSA control and the 2 0M NaCl fraction The very low absorbances measured may have led to such minor discrepancies

As the readings were very low, it was decided to look at the biological activity of citrate in the presence and absence of the unbound faction If citrate was the active factor, it was possible that recombination of citrate and the unbound fraction could result in recovery of activity As no concentration of citrate was mentioned by Kane (1990), a wide band of concentrations were assayed The results are shown in Table 3 5 4 10 2

The results show that citrate alone at increasing concentrations became increasingly inhibitory In two of three experiments 75% to 80% inhibition was seen at 1000μg/ml The unbound fraction had little or no activity and when combined with citrate, a similar trend was seen to that for the citrate alone

Table 3.5.4.10.2 Growth response of NRK cells to citrate

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 $\pm$ 4 63	100 0 $\pm$ 8 16	100 0 $\pm$ 8 94
+ 1μg/ml C	99 27 $\pm$ 5 47	101 5 $\pm$ 6 15	112 8 $\pm$ 10 3
+ 10μ/ml C	89 23 $\pm$ 5 29	101 5 $\pm$ 5 97	100 9 $\pm$ 9 29
+ 100μg/ml C	74 09 $\pm$ 4 56	98 50 $\pm$ 4 48	77 98 $\pm$ 4 38
+ 1000μg/ml C	38 50 $\pm$ 2 74	97 00 $\pm$ 5 97	36 62 $\pm$ 1 08
+ 2mg/ml Unbound Fr	114 8 $\pm$ 5 10	102 9 $\pm$ 10 4	81 67 $\pm$ 12 3
+ 1μg/ml C	107 3 $\pm$ 5 84	100 0 $\pm$ 4 48	80 38 $\pm$ 9 87
+ 10μg/ml C	107 7 $\pm$ 6 57	102 9 $\pm$ 5 97	71 44 $\pm$ 4 72
+ 100μg/ml C	82 48 $\pm$ 6 57	92 54 $\pm$ 8 95	49 09 $\pm$ 3 41
+ 1000μg/ml C	27 37 $\pm$ 5 47	89 55 $\pm$ 5 97	31 49 $\pm$ 1 75

Results are expressed as the average percentage growth relative to control (1% DHS) $\pm$ standard deviation (n=8) Abbreviations C = citrate, Unbound Fr = unbound fraction from Run 11

3.5.4.11 Determination of the phospholipid content of HS fractions

To investigate the possibility that the activity of the 0 5M NaCl fractions was due to phospholipids, a biochemical determination kit was obtained from bioMerieux (Cat No 61491) This kit was designed to determine phospholipids which liberate choline on hydrolysis by phospholipase D The results are shown in Table 3 5 4 11 for Run 7 Samples were left in a concentrated form in order to have the best chance of obtaining values in the linear part of the assay system

Table 3.5.4.11 Phospholipid levels in HS fractions (mM)

RUN	BSA	UNBOUND FRACTION	BUFFER WASH	0 5M NaCl WASH	1 0M NaCl WASH	2 0M NaCl WASH
2	0 850	0 430	0 633	0 547	0 601	0 742
3	0 330	0 164	0 094	0 609	1 040	0 648
4	0 781	0 742	0 476	0 664	0 531	0 445
5	1 191	0 671	0 355	0 497	0 308	0 299
6	---	0 089	0 126	0 284	0 576	0 458
8	1 089	0 130	0 071	0 702	0 522	0 607

The linearity of this colorimetric assay was 0 to 10mM In the first set of readings (not shown), the values obtained were so low that the assay was repeated This time 100μl sample instead of 10μl of sample was added (100μl of standard and blank were also used) The results shown above were at the lower end of the linearity range No trend was seen and the combination of values for each of the fractions was greater than that found in the BSA control In addition to the low level of phospholipid present, the fact that the colour formed was a pinky-red meant that the medium could have been interfering with the results (this would explain why the combination of values from each of the fractions did not equate with the value obtained for the control BSA)

3.5.4.12 Lyophilisation of BSA and 0.5M NaCl Fraction

It was considered that further purification of the growth stimulating activity might be effected by HPLC However, in order to use the HPLC, it was necessary to see if the activity of the 0 5M NaCl fraction was retained after lyophilization Lyophilization was carried out as described in section 2 14 The BSA and 0 5M NaCl fractions were first desalted and then split into 2 Both samples were lyophilized overnight One sample was dissolved back up in ATCC The second was dissolved into a solution of 80% acetonitrile (ACN) and 0 1% trifluoroacetic acid (TFA) This was the highest concentration of acetomtrile that samples would be exposed to during HPLC fractionation This sample was lyophilized again and dissolved into ATCC

The results are shown in Table 3 5 4 12 1 For the BSA there was a decrease from the untreated BSA to the lyophilized BSA (30-38%) and a further decrease in activity for the sample exposed to ACN/TFA This sample was extremely acidic when reconstituted, as indicated by the yellow colour of the sample when it was reconstituted in ATCC medium For the 0 5M NaCl fraction, there was no decrease for the initial lyophilization However, there was a decrease when the sample was exposed to ACN/TFA (40 - 50%) before lyophilisation

These results indicated that both the BSA and 0 5M NaCl fractions were sensitive to lyophilization after exposure to acetomtrile The BSA appeared to be more susceptible to loss of activity than the 0 5M NaCl fraction The acidic nature of the BSA solution indicated that the low activity may have been due to residual ACN/TFA which may not have fully lyophilized from the sample It would appear that both would lose some activity if HPLC was used as a means of trying to isolate out the active fraction

Table 3.5.4.12 Effect of lyophilisation on the activity of BSA and the 0 5M NaCl fraction

SAMPLE	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 8 22	100 0 ± 7 89	100 0 ± 12 0
BSA control	324 3 ± 21 9	280 0 ± 20 0	267 6 ± 17 9
BSA lyo	204 4 ± 14 6	174 4 ± 9 81	189 2 ± 18 4
BSA lyo (Acn)	79 49 ± 11 6	73 30 ± 13 3	69 37 ± 7 72
0 5M NaCl con	255 4 ± 29 9	240 0 ± 21 5	189 2 ± 6 89
0 5M NaCl lyo	262 8 ± 12 3	228 9 ± 20 1	189 2 ± 18 2
0 5M NaCl lyo (Acn)	180 2 ± 22 2	138 8 ± 15 4	97 30 ± 13 0

Results show the average growth relative to control (1% DHS) ± standard deviation (n=8) Dye elution was used as the end point for assays Abbreviations lyo = lyophilised, lyo (Acn) = lyophilised in an 80% Acetonitrile(0 1% TFA) solution

3.5.4.13 Thymidine incorporation assay

The purpose of this experiment was to establish whether the 0 5M NaCl fraction could stimulate the incorporation of exogenous thymidine into cellular DNA, often taken as a marker for DNA synthesis NRK cells were set up in serum-free medium (section 3 1 6) The cells were left for 18 hours before increasing dilutions of the 0 5M NaCl fraction and the control BSA were added to the serum-free medium 4 hours later radio-labelled thymidine was added After 12 hours the amount of ^{3}H incorporated into the cells was determined using a scintillation counter The results for three separate experiments are shown in Table 3 5 4 13 1

Table 3.5.4.13.1 Results for thymidine incorporation assay

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
Basal Medium	130 9 ± 14 4	161 3 ± 7 30	97 6 ± 12 9
SFM	100 0 ± 10 6	100 0 ± 21 3	100 0 ± 9 16
+ 0 1mg/ml BSA	93 26 ± 8 98	523 4 ± 94 6	197 1 ± 43 6
+ 0 5mg/ml BSA	129 8 ± 3 44	227 3 ± 39 9	207 2 ± 20 5
+ 1 0mg/ml BSA	94 98 ± 16 1	195 3 ± 11 8	212 4 ± 18 8
+ 2 0mg/ml BSA	145 4 ± 66 6	537 4 ± 63 4	140 3 ± 8 43
+ 0 1mg/ml 0 5M NaCl	157 6 ± 12 5	130 9 ± 17 3	144 5 ± 21 0
+ 0 5mg/ml 0 5M NaCl	142 0 ± 23 6	168 1 ± 12 6	123 6 ± 22 2
+ 1 0mg/ml 0 5M NaCl	227 1 ± 16 5	602 9 ± 1 85	86 32 ± 10 3
+ 2 0mg/ml 0 5M NaCl	351 7 ± 26 9	368 5 ± 31 2	34 80 ± 7 46

Results are shown as the average percentage growth relative to control (SFM) ± standard deviation (n=4) The serum-free medium contains 10μg/ml insulin and 1 39μg/ml Fe_2SO_4 Samples from Runs 7, 8 and 11 were used in assays 1 to 3 respectively

The results show that increasing concentrations of the 0 5M NaCl fraction caused increased incorporation of exogenous thymidine into DNA in two of three assays The response in the third assay showed that at increasing concentrations of 0 5M NaCl fraction, decreasing activity at 0 1 to 0 5 mg/ml with inhibition seen with the higher concentrations tested For the BSA control, the response was not linear with respect to increasing albumin concentration, however the best growth was seen the highest concentration tested (2 0mg/ml) in two of the three experiments The difference in the extent of stimulation between the Assay 2 and the other assays may have been due to the initial cell density The second assay was set up at $4x10^{4}$ while the first and third were set up at $2x10^{5}$ It was also interesting to note that the basal

medium allowed for more incorporation of exogenous thymidine into DNA synthesises than the serum-free medium

Taken together, the results from the thymidine incorporation assays appeared to be quite variable The 0 5M NaCl fraction allowed for greater stimulation of exogenous thymidine into the DNA than BSA in two of three assays

3.5.4.14 Effect of 0.5M NaCl fraction on NRK cells in SFM

With the positive stimulation observed with the BSA and 0 5M NaCl fraction for the NRK cells in the thymidine incorporation assay, it was decided to see if the factors increased growth of the cells over an extended period in SFM

Previous assays using BSA showed growth inhibition of NRK cells under serum-free conditions (section 3 1 7 3) In the thymidine incorporation assay the factors to be tested were not added until 18hours after plating It was thought that the inhibitory action may have been due to the albumin interfering with attachment of the cells With this in mind, cells were set up at $1x10^4$ cells per well in 96-well plates in the SFM After 18 hours, the BSA and 0 5M NaCl fractions were added in serum-free medium The cells were incubated for 4 more days The assays was read using acid phosphatase as the end point The results are shown in Table 3 5 4 14

Table 3.5.4 14 Results for serum-free assay

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3	ASSAY 4
SFM	100 0 ± 3 78	100 0 ± 3 78	100 0 ± 8 40	100 0 ± 6 20
+ 0 1mg/ml BSA	-------	109 7 ± 4 99	201 6 ± 9 16	110 7 ± 7 71
+ 0 5mg/ml BSA	77 59 ± 8 37	100 0 ± 4 99	43 08 ± 4 93	90 48 ± 11 9
+ 1 0mg/ml BSA	64 94 ± 5 52	100 0 ± 7 23	36 50 ± 4 68	79 80 ± 6 18
+ 2 0mg/ml BSA	44 83 ± 3 45	109 1 ± 8 39	------	74 80 ± 12 6
+ 0 1mg/ml 0 5M NaCl	------	92 73 ± 1 21	209 4 ± 4 15	71 62 ± 8 01
+ 0 5mg/ml 0 5M NaCl	66 66 ± 5 63	83 03 ± 3 64	36 70 ± 4 61	64 91 ± 5 80
+ 1 0mg/ml 0 5M NaCl	61 58 ± 7 29	79 39 ± 4 14	25 5 ± 2 55	60 70 ± 5 90
+ 2 0mg/ml 0 5M NaCl	33 33 ± 5 19	76 12 ± 4 06	-------	33 33 ± 5 19

Results are shown as the average percentage growth relative to control (SFM) ± standard deviation (n=8) The serum-free medium contains 10μg/ml insulin and 1 39μg/ml Fe_2SO_4 Samples from runs 7, 8, 11 and 12 were used for assays 1 to 4 respectively

The results show that BSA and 0 5M NaCl fractions caused increasing inhibition at increasing concentrations For each experiment, the 0 5M NaCl fraction was more inhibitory than the BSA At the lowest concentration tested, the 0 5M NaCl fraction and BSA were stimulatory in only assay 3 It may have been that the concentrations used in low serum-supplemented medium were too high in a serum-free medium

Comparing the results from the thymidine incorporation assay and the serum-free growth assay, it can be seen that the incorporation of exogenous thymidine into DNA does not necessarily mean cell growth will follow It was also interesting to note that while the 0 5M NaCl fraction was slightly better at stimulating thymidine incorporation, it was more inhibitory to the cells over an extended period of time (7 days) in SFM

3.5.4.15 pH Stability

The purpose of these experiments was to establish the stability BSA and the 0 5M NaCl fraction at a range of pH values Two separate experiments were carried out for the BSA (two assays per experiment) and one experiment was carried out with the 0 5M NaCl fraction Dye elution was used as the end point for the BSA experiment and cell number was used as the end point for the 0 5M NaCl fraction Stocks of BSA and 0 5M NaCl fractions were exposed to various pH values by slow addition of 10μl volumes of 1M NaOH or 1M HCl with constant stirring The samples were then incubated at 37°C for 2 hours The pH was then readjusted slowly to 7 4 The results are shown in Tables 3 5 4 15 1 and 2

The results show that exposure to a range of pHs had little or no effect in the first experiment and some loss of activity in the second experiment In the first assay, no loss of activity was seen except for pH 12 where a 10% loss of activity was observed In the second experiment, a small decrease in activity was seen, 14 - 23% in assay 1 and 7 - 15% in assay 2 The pH control for BSA, *i e* the sample in which the pH was adjusted to pH 7 40 for the incubation period, showed a similar loss in activity in relation to the untreated BSA, as that seen in the samples exposed to pH 12 or pH 2 6 This would suggest that a very small portion of the BSA's activity appeared to be susceptible to acid/base but no more than 23%
The 0 5M NaCl fraction appeared to be slightly more sensitive to the effects of pH with losses of 35 5 - 44 7% in the first assay and 25 3 - 36 2% in the second assay The loss in activity was similar irrespective of the pH that the samples were exposed to

Table 3 5 4.15.1 Effect of pH on the bioactivity of BSA

VARIABLES	EXPERIMENT 1		EXPERIMENT 2	
	Assay 1	Assay 2	Assay 1	Assay 2
1% DHS	100 0 ± 5 63	100 0 ± 5 43	100 0 ± 2 91	100 0 ± 6 30
Con BSA	269 0 ± 12 2	263 7 ± 9 90	345 0 ± 26 0	311 0 ± 12 3
pH 2 6	281 1 ± 16 6	271 4 ± 19 2	266 9 ± 19 6	263 4 ± 20 7
pH 4 3	282 4 ± 19 4	276 7 ± 15 5	283 0 ± 17 4	278 2 ± 14 9
pH 6 0	260 0 ± 21 1	258 0 ± 15 9	274 0 ± 12 9	281 2 ± 24 2
pH 7 4	252 7 ± 12 8	246 8 ± 15 5	290 1 ± 17 0	279 8 ± 16 1
pH 8 8	266 6 ± 14 4	248 9 ± 14 4	296 5 ± 13 2	288 8 ± 13 1
pH 10 0	262 0 ± 15 5	270 3 ± 21 9	287 1 ± 10 6	279 0 ± 13 1
pH 11 0	275 0 ± 17 8	266 6 ± 18 1	290 0 ± 13 5	283 9 ± 21 4
pH 12 0	241 8 ± 30 0	239 7 ± 13 3	280 0 ± 19 6	271 3 ± 15 4

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Crystal violet dye elution was used as the end point of experiments

Table 3.5.4.15.2 Effect of pH on the bioactivity of 0 5M NaCl fraction

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 10 9	100 0 ± 6 28
0 5M NaCl control	352 1 ± 15 7	335 6 ± 37 3
pH 2 6	194 8 ± 25 6	250 6 ± 29 1
pH 4 3	227 3 ± 32 9	248 6 ± 8 72
pH 8 8	218 8 ± 20 7	224 6 ± 28 4
pH 10 0	196 6 ± 18 0	238 4 ± 18 8

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3) Cell number was used as the end point for experiments

These results taken together would indicate that while there was only an average of 14% loss of activity on exposure of BSA to acid or base, there was about 35% loss in activity of the 0 5M NaCl fraction It may be that the albumin confers some protection for the active component and that loss of much of the protein during affinity chromatography renders the 0 5M NaCl fraction more susceptible to the action of acid or base

For pH 2 6, variable (37 6 and 63%) loss in activity occurred These results would support the findings while studying the effect of pepsin, that pH 2 6 can result in loss of activity of the 0 5M NaCl fraction

3.5.4.16 Dialysis of BSA and the 0.5M NaCl Fraction

In order to see if the activity associated with the BSA and 0 5M NaCl fraction was stable upon dialysis, samples of both were dialysed against 200X volume of ATCC medium (mol wt cut off = 10,000) The results are shown in Table 3 5 4 16

Table 3.5.4.16 Effect of dialysis on the activity of BSA and the 0 5M NaCl fraction

VARIABLES	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 12 4	100 0 ± 4 04
Control BSA	199 9 ± 10 2	232 2 ± 27 0
0 5M NaCl	194 3 ± 14 7	203 8 ± 21 6
Dialysed ATCC control	122 9 ± 19 7	121 6 ± 27 8
Dialysed 0 5M NaCl	163 9 ± 11 4	194 3 ± 5 71
Dialysed BSA	172 1 ± 17 0	168 6 ± 8 57

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3) Cell number was used as the end point for experiments

The results show that some slight loss of activity was seen for both the 0 5M NaCl fraction and the BSA In the first assay, there was a 28% loss in activity while in the second assay, dialysis of BSA resulted in a 48% loss in activity while the 0 5M NaCl fraction lost 10 - 30% activity The ATCC control which was dialysed showed slightly increased growth (21 - 23%) relative to the ATCC control which was not dialysed This difference in growth stimulation was negligible when the standard deviations were taken into account If however, this ATCC control was used as a basis, 34 - 47% activity was lost from the 0 5M NaCl fraction and 36 - 57 5% was lost for the BSA The BSA, 0 5M NaCl and ATCC control were dialysed in separate containers, so the increase in activity in the ATCC control could not be as a result of the loss in activity of the 0 5M NaCl fraction or the BSA This would indicate that some inhibitory factor was lost during dialysis of ATCC

3.5.4.17 Reapplication of 0.5M NaCl fraction to the HS column

Up until now, it was assumed that the protein in the 0 5M NaCl wash was specifically bound to the heparin sepharose and specifically eluted off with the 0 5M NaCl wash However, it was possible that the protein was residually in the column and that more extensive washing with the

Na_2HPO_4 buffer may have removed it In order to determine the specificity of binding of the 0 5M NaCl fraction, a sample of the 0 5M NaCl fraction was reapplied to the HS column This fraction was treated as if it were the unfractionated BSA Two separate experiments were carried out The results of the second experiment are shown in Table 3 5 4 17 1

The results for the first reapplication experiment were disappointing in that the level of activity of the 0 5M NaCl fraction from the first application was very low and all the factors appeared inhibitory on the reapplication However, the protein profile showed that the only detectable amount of protein which was measured in the reapplied sample was eluted off in the 0 5M NaCl wash, supporting the idea that the activity was indeed specifically eluted off in the 0 5M NaCl wash

In the second reapplication experiment, good activity was seen in the 0 5M NaCl wash in both applications The results are shown in Table 3 5 4 17 2 The activity in the 0 5M NaCl wash on reapplication was better than that in the first application Inhibition ranging from 13% to 70 0% was seen in the remaining fractions after the reapplication

Table 3.5.4 17.2 Reapplication of the 0 5M NaCl fraction to HS column

VARIABLES	BSA	0 5M NaCl	BSA	0 5M NaCl
1% DHS	100 0 ± 7 31	100 0 ± 17 9	100 0 ± 14 7	100 0 ± 12 9
Control BSA	214 9 ± 56 9	193 8 ± 9 23	262 3 ± 20 7	196 3 ± 25 1
Unbound fraction	63 79 ± 12 2	30 00 ± 3 99	69 45 ± 11 9	82 27 ± 6 12
Buffer Wash	31 00 ± 2 44	54 23 ± 1 63	42 11 ± 11 9	65 38 ± 1 02
0 5M NaCl Wash	199 0 ± 13 1	290 8 ± 26 0	216 7 ± 10 3	278 8 ± 29 5
1 0M NaCl Wash	102 3 ± 15 4	86 92 ± 8 74	114 7 ± 19 3	62 50 ± 8 33
2 0M NaCl Wash	83 30 ± 6 97	46 92 ± 9 32	110 8 ± 7 40	39 42 ± 10 9

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Cell number was used as the end point for experiments The protein concentrations for the samples are BSA = 7 6mg/ml, unbound fraction = 6 0mg/ml, buffer wash = 1 163mg/ml, 0 5M NaCl fraction = 0 38mg/ml and all the samples for the reapplication had no detectable protein concentrations

However, although the protein concentration of the initial BSA solution was gravimetrically, 15mg/ml, spectrophotometrically the concentration was only 7 6mg/ml As a result of the low initial protein concentrations, no protein was detected in the reapplied samples

The results show that for heparin sepharose the activity of the 0 5M NaCl fraction specifically eluted in the 0 5M NaCl wash It also shows that further purification of the 0 5M NaCl fraction resulted in increased activity in the second assay It may be that reapplication of the 0 5M NaCl removed some factor(s) that maybe inhibitory

3.5.4.18 Application of heat-shock albumin to heparin sepharose

As the 'heat-shock' albumin (Figure 3 5 2 4) showed greater stimulation than the Cohn fraction V albumins, it was of interest to subject this albumin to HS chromatography to see if the same trend were observed with BSA fraction V (A4919) The results are shown in Table 3 5 4 18

Table 3 5 4 18

FRACTION	ASSAY 1	ASSAY 2
1% DHS	100 0 ± 10 2	100 0 ± 7 70
+ BSA Con	485 5 ± 26 1	181 3 ± 16 5
+ Unbound Fraction	182 5 ± 12 7	95 10 ± 8 41
+ Buffer Wash	115 9 ± 26 9	68 61 ± 5 18
+ 0 5M NaCl	250 9 ± 21 8	136 1 ± 2 43
+ 1 0M NaCl	203 6 ± 52 7	109 8 ± 8 10
+ 2 0M NaCl	158 0 ± 23 2	91 60 ± 6 06

Results are shown as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Cell number was used as the end point for experiments

The results show very different trends between the two experiments In the first assay, very good stimulation was seen in all fractions except the buffer wash The cell growth in this assay was very low In the second assay, the extent of stimulation was very low, with only a fraction of the activity being isolated in the 0 5M NaCl fraction

The presence of activity in the other fractions obtained with this albumin in assay 1, may indicate that for the heat-shock albumin, a number of factors are contributing to the activity, including the factor eluted in the 0 5M NaCl wash The amount of activity isolated in the 0 5M NaCl fraction was 44% of the control according to both assays So it would appear that the heat-shock albumin would not be a more plentiful source of the active factor associated with the 0 5M NaCl fraction than the albumin used in these studies

3.5.5 BSA AND REVERSE PHASE - HPLC

Congote (1987) found the contaminating factor associated with BSA which stimulated thymidine incorporation into rat erythroid cells in serum-free medium to be an erythrotropin-like factor Reverse phase HPLC was used as the initial purification step in isolating the active factor This technique was used to see if the BSA fraction active for NRK cells was related to the erythrotropin-like factor

A C18 μbondapak column was used with a linear gradient of 20% to 80% vol/vol acetonitrile (ACN) with 0 1% trifluoroacetic acid (TFA) in ultrapure H_2O The gradient program was run for 26 minutes at a flow rate of 1 0ml/min A solution of 200mg/ml BSA (A4919, lot 110H 04635) was made up and 50μl was added to the 20μl injection loop per run An additional 5-6 minutes were allowed for all peaks to elute off the column Fractions were lyophilized to remove the acetonitrile before testing for bioactivity

3.5.5.1 Protein Elution Profiles

In order to get enough sample to test the bioactivity of the fractions, it was necessary to carry out 26 runs on the HPLC column The elution profiles of absorbance at 280nm from the 26 runs showed very consistent results One of the elution profiles is shown in Figure 3 5 5 1 For all runs a small blip occurred at 27 - 30%ACN The main protein peak eluted off in fractions 50 - 60%ACN There was always a small lip in front of the main peak, after which, the main peak rose abruptly and then showed a tailing effect as the absorbance returned to base level Three small peaks were present at the end of the gradient varying between fractions 13, 14 and 15 and fractions 12, 13 and 15

One sample of BSA-coproporphyrin 1 mixture was run and the detection at 400nm was measured Coproporphyrin was applied as Congote found the erythrotropin-like factor to elute at the same point as coproporphyrin A dip and peak was seen around 30%ACN Another peak was seen at 50%ACN which coincided with the main albumin protein peak
Comparison of the elution profile (Figure 3 5 5 3 2) obtained with Congote's elution profile showed differences in the position of the main albumin peak and the coproporphyrin peak In these studies, the major protein peak was eluted off at 50% ACN, as compared to a value of 30% ACN reported by Congote The elution profile obtained at 400nm with the coproporphyrin sample, showed one main peak at 27% ACN followed by a trough, with a

second peak at 55 - 60% ACN corresponding to the main albumin peak This peak may have been due to interference from the albumin in the sample which necessitated running coproporphyrin alone

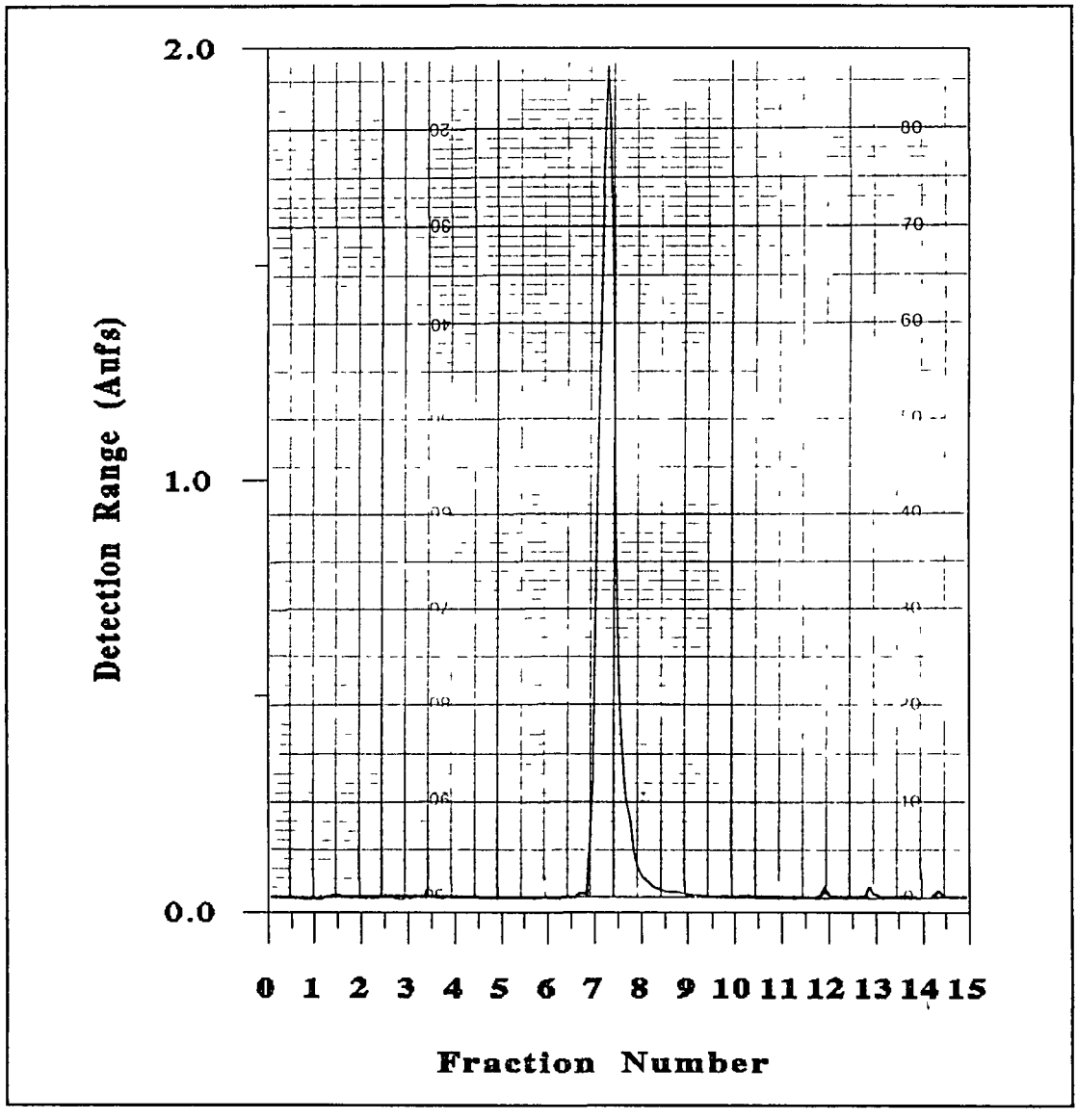


Figure 3.5.5.1 Elution profile for BSA (A4919) sample on C18 μBondapak column The abosrbance was detected at 280nm The chart speed was set at 5mm/min with a detection range of 0 - 2 0 Aufs

3.5.5.2 Bioactivity of fractions

Fractions of 2ml volumes were collected (11 runs on column) The samples were frozen at -70°C immediately after elution from the column until all samples were collected The samples were then lyophilized and pooled together When all the samples were lyophilized, a white residue was left in fractions 7, 8 and 9 (the greatest volume being in 8) No problem was encountered on reconstitution of fractions 7 - 9, however the media turned a straw-yellow colour similar to that of the untreated albumin For the rest of the samples no residue was visible and on reconstitution no variation in colour occurred The samples were tested at an albumin(equivalent) concentration of 5mg/ml Assays were set up in 24-well plates and 96-well plates to look at the biological activity Dye elution was used as the end point of experiments for assays set up in 96-well plates and cell number was used as the end point for assays carried out in 24-well plates The results are shown in Table 3 5 5 2

For both control end points, the 20% and 80% Acetonitrile samples showed no effect on growth The lyophilized BSA control was not as active as the original BSA sample (section 3 5 4 16) The only fractions which consistently showed stimulation for both end points (dye elution and cell number) were fractions 6 and 13 Fraction 13 appeared most significant with 60% stimulation above the control (1% DHS) only for cell number The extent of stimulation was not as high when dye elution was used as the end point Fraction 6 came off the column just before the major protein peak and small peaks were observed in 12, 13 and 14 For both end points, inhibition occurred in a band between fractions 7 and 9, the fractions in which the major protein was eluted off

In comparison to the untreated BSA control, 50 - 80% of the activity was retained upon lyophilization Relative to the lyophilized BSA control 8 5 - 17% activity was retained in fraction 6 using dye elution as the end point and 37% activity was retained when cell number was the end point When compared to the untreated BSA control, the activity was reduced to 7 - 10% for dye elution and 20% for cell number as end points For fraction 13, less than 10% and 25% of the activity of the untreated BSA was observed for dye elution and cell number as end points respectively

The loss of activity may have been due to instability of the active fraction in the solvents or it may be that the active fraction was isolated from the albumin and without the albumin, was not stimulatory

There may also have been some loss of activity in the albumin itself when exposed to increasing acetonitrile concentrations In addition, it took 36 hours for the samples to be lyophilized From previous results it was known that lyophilization did reduce the activity of the albumin but not the 0 5M NaCl fraction On exposure to acetonitrile before lyophilization, both showed reduced stimulation (section 3 5 4 12)

Table 3 5 5.2 Bioactivity of HPLC fractions

VARIABLES	DE/ ASSAY 1	DE/ ASSAY 2	CN/ ASSAY 1
1% DHS	100 0 ± 8 16	100 0 ± 11 9	100 0 ± 17 6
+ 20% ACN Con	109 9 ± 12 3	99 26 ± 4 75	104 4 ± 16 8
+80% ACN Con	104 3 ± 12 5	96 29 ± 9 99	100 0 ± 26 7
+ BSA Con	395 5 ± 23 6	303 7 ± 27 2	347 7 ± 23 6
+ BSA (lyo)	335 2 ± 14 1	223 3 ± 19 0	225 0 ± 19 3
+ fraction 1	117 7 ± 6 33	103 4 ± 6 82	95 45 ± 11 8
+ fraction 2	100 7 ± 2 67	88 57 ± 6 70	93 18 ± 15 7
+ fraction 3	102 4 ± 6 59	99 05 ± 12 2	106 1 ± 27 8
+ fraction 4	106 7 ± 10 0	90 61 ± 10 4	129 5 ± 29 7
+ fraction 5	90 00 ± 5 16	94 28 ± 8 57	106 8 ± 20 8
+ fraction 6	120 0 ± 10 0	120 9 ± 14 9	146 7 ± 20 2
+ fraction 7	76 66 ± 4 71	40 95 ± 6 68	40 00 ± 10 0
+ fraction 8	70 00 ± 6 66	57 62 ± 5 83	43 33 ± 15 3
+ fraction 9	93 83 ± 7 28	86 73 ± 7 63	73 33 ± 10 4
+ fraction 10	100 6 ± 5 93	99 26 ± 9 14	66 66 ± 27 5
+ fraction 11	122 9 ± 7 12	103 3 ± 12 8	76 66 ± 5 77
+ fraction 12	118 5 ± 10 2	107 9 ± 6 64	131 1 ± 10 2
+ fraction 13	124 4 ± 7 68	118 9 ± 12 6	163 3 ± 14 1
+ fraction 14	104 3 ± 12 7	105 7 ± 7 97	122 2 ± 13 9
+ fraction 15	113 1 ± 8 28	96 75 ± 7 96	131 1 ± 19 2

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8 for DE and n=3 for CN) Abbreviations DE = dye elution, CN = cell number, BSA con = untreated BSA at 5mg/ml, BSA (lyo) = BSA which has been lyophilized, tested at 5mg/ml, 20% and 80% ACN con = solvent control to ensure no residue from the solvent affected growth (20% ACN at beginning and 80% at end of gradient)

3.5.5.3 Elution Profile of Coproporphyrin

On the basis of the results obtained from the first set of runs on the HPLC, it was necessary to establish whether or not the coproporphyrin was eluting off with the small amount of activity seen in fractions 6 and 13 A lower detection level was used both with and without albumin present in the solution

When the elution profile of the albumin was compared this time around with the profile obtained in the first set of experiments, a subtle change was observed The blip which appeared to correspond with the coproporphyrin was now eluting off at 50 - 60mm as opposed to 27 - 30mm The main protein peaks were coming off in fraction 7 (as opposed to 8 in the last set) and the three small peaks were coming off in fractions 12 - 14

The results for the detection of coproporphyrin at 400nm are shown in Table 3 5 5 3 1 For the different runs, a variety of peaks and troughs were present The table shows the retention time for the various peaks at 400nm both with and without albumin

Table 3.5.5.3 1 Elution profiles (expressed as mm from injection point)

Run	Sample	Det Range (Aufs)	PEAKS					
			1	2	3	4	5	6
1	20mg/ml Albumin + CoP	0 0 - 0 01	24 5	31 5	38 5	68 5	90 5	
2	20mg/ml Albumin + CoP	0 0 - 0 02	24 5	31 5		69 0		110*
3	200mg/ml Albumin + CoP	0 0 - 0 02		32 0		68 5	90 5	110*
4	500μg/ml CoP	0 0 - 0 1		32 0		72 0		110*
5	500μg/ml CoP	?		31 5		73 0		110*

Abbreviations Det = detection range (aufs), CoP = coproporphyrin, unless stated at 0 1μg/ml, 110* indicates the presence of erratic absorbances after this retention time

The grouping of retention times showed two peaks common to all runs, peak 2 at 31 5 - 32mm (34 - 34 8% ACN) and peak 4 at 68 5 - 73mm (51 6 - 53 7% ACN) Peak 2 was followed by a slight trough Peak 4 was not symmetrical and had a slight tail, similar to the main albumin peak.

These peaks corresponded to the small blips seen at 12 - 17mm in the first set of runs and to the main protein peak They showed a similar shape to the coproporphyrin detected in run 8 from the first set of experiments (Figure 3 5 5 3 1) However, there was a discrepancy in the retention times At a later stage, the coproporphyrin was run alone at 500μg/ml The peak and trough appeared again but the peak was eluted of at 17mm

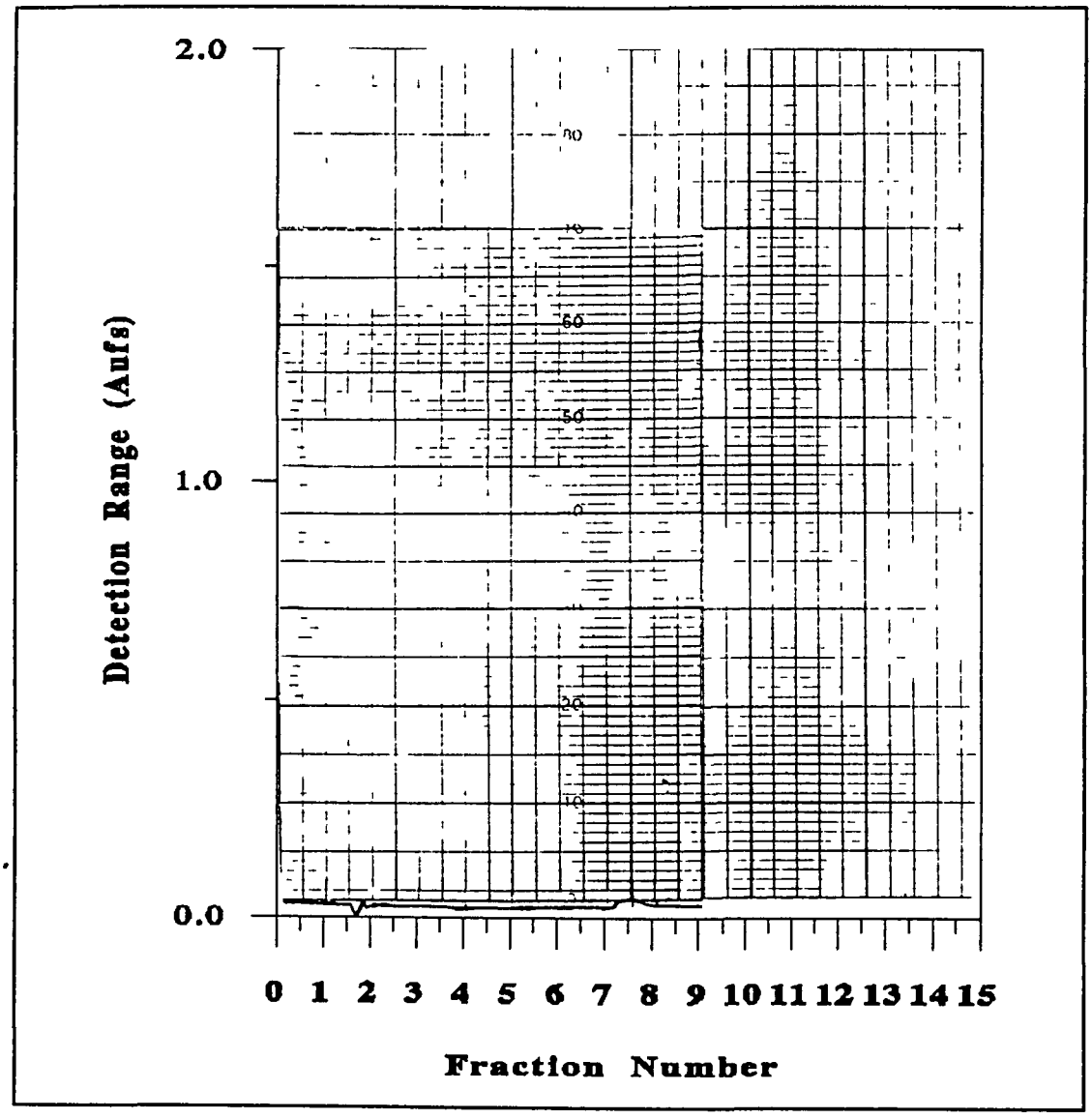


Figure 3.5.5.3.1 Elution profile for Coproporphyrin I (0 2μg/ml) sample on C18 μBondapak column The absorbance was detected at 400nm The chart speed was set at 5mm/min with a detection range of 0 - 2 0 Aufs

Peak 4 appeared to have a slightly longer retention time in the absence of albumin Peak 4 coincided with the main protein peak for the BSA and coincided with the second protein peak obtained by Congote Peak 2 had a slightly greater retention time than the first peak obtained by Congote. There was some disparity between our chromatograms and those obtained by

Congote However, our results show that the two most active fractions (fractions 6 and 13) did not coincide with the two most common peaks observed with coproporphyrin Figure 3 5 5 3 2 shows the chromatogram for run 2

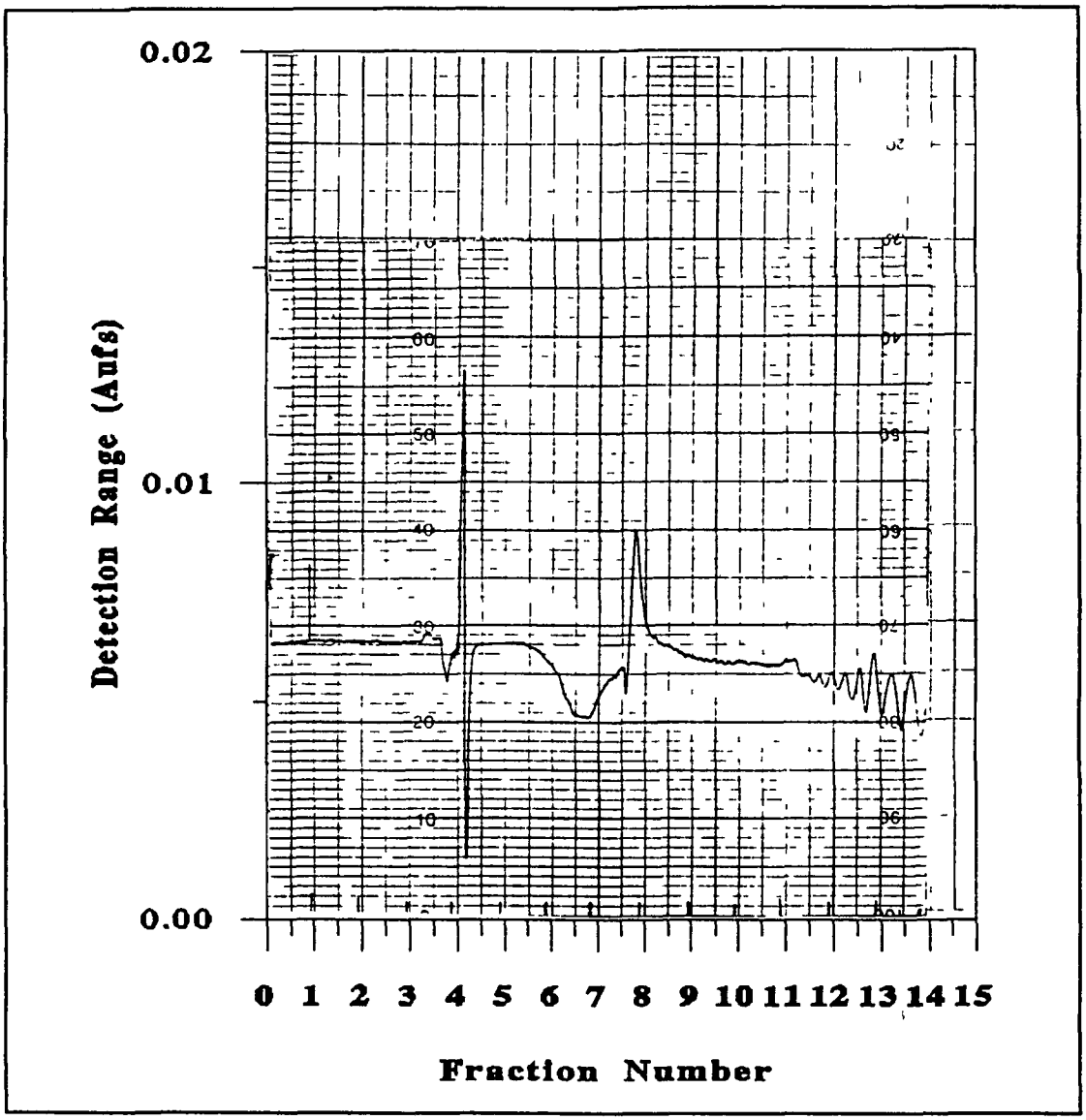


Figure 3.5.5.3.2 Elution profile for 20μg/ml Coproporphyrin I on C18 μBondapak column The abosrbance was detected at 400nm The chart speed was set at 5mm/min with a detection range of 0 - 0 02 Aufs

The 0 5M NaCl fraction was desalted, lyophilized and applied to the RP-HPLC column The resulting chromatogram (Figure 3 5 5.3.3) shows the following information A peak and trough was seen in fraction 4 (whose shape coincided with the first coproporphyrin peak) There was a small peak between fractions 5 and 6, that was not present with the albumin. There was also another peak at fractions 6 - 7 before the main peak This was probably the small lip seen at the beginning of the main albumin peak which would not be seen at a detection range with low sensitivity (0 0 - 2 0Aufs, for the BSA) The three small peaks present at the end of the gradient for the albumin, occurred in the same position for the 0 5M NaCl fraction and BSA, however the size of the three peaks relative to the main peak was much larger

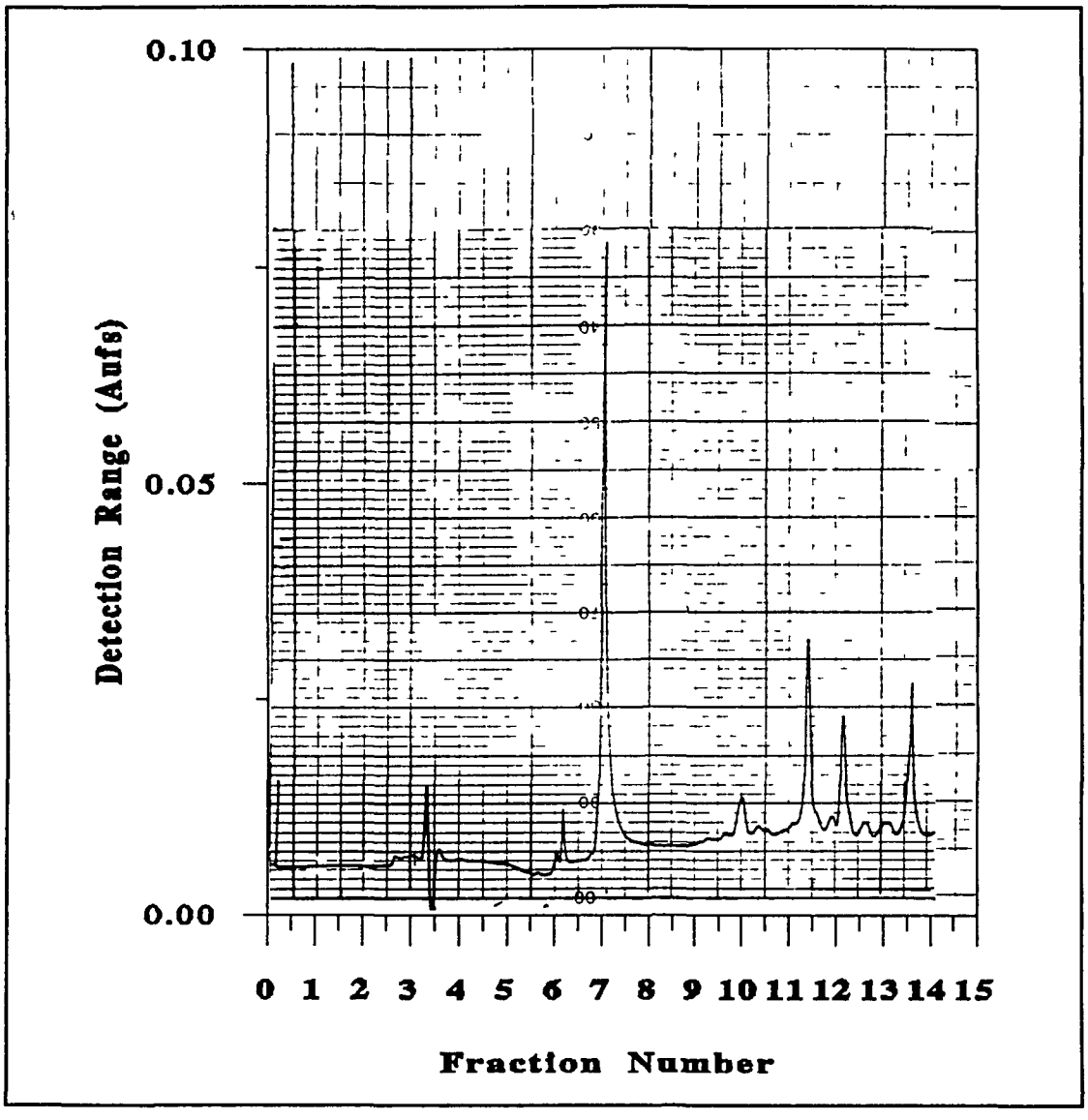


Figure 3.5.5.3.3 Elution profile for 0 5M NaCl fraction (20mg/ml) on C18 μBondapak column at 280nm abosrbance The chart speed was set at 5mm/min with a detection range of 0 - 0 1 Aufs.

3.5.5.4 Study of the three small peaks at the end of the gradient

On the basis of the results in section 3 5 5 3, the intention was to run BSA and 0 5M NaCl samples with a view to isolating the 3 small peaks after the main protein peak, to see if the activity was associated with one in particular It was intended to do this by changing the gradient to allow greater separation between the bands and/or to collect smaller fractions off the column

It was also intended to try and overload the column, to see if a protein profile like that of Congote's was achieved

However, when the gradient was run again without albumin injection, three peaks were observed in the area of the gradient that most of the activity was seen These corresponded to the small blips seen when running the albumin where the detection range was set at 0 0 to 2 0 Aufs On the control run without albumin injection, the detection was set at 0 0 to 0 05 Aufs It was initially thought that the peaks may have been due to residual protein on the column

To get rid of these peaks the following were tried

1/ After several runs with the gradient, there was no decrease in the height of the peaks

2/ 50ml 100% methanol was run through column After an initial change in absorbance due changing mobile phase no additional peaks appeared

3/ 100% acetomtrile was run through the column No peaks observed in the first run after, however, in following runs the peaks were present

4/ As the peaks were eluting off the column at about 80% ACN, the column was run at 80% for an extended period No additional peaks were seen

5/ The column was stored column in iso-propanol over night to see if this would remove contaminants No change in elution profile was seen

6/ The recommended cleaning procedure as described in the manual accompanying the column was used It incorporated washes of ultrapure H_2O, methanol, injections of DMSO, methanol and finally ultrapure H_2O No change in elution profile was seen

7/ The column was washed through with many column volumes of ultrapure H_2O Again no change in the elution profile was seen

8/ The guard column filter was replaced but no change in the elution profile was seen

9/ The guard column was removed altogether but no change in the elution profile occurred

10/ The gradient was run with just guard column This time no peaks were seen

11/ A different source of acetonitrile was tried Again no change in the elution profile was seen

12/ A Novopak C_{18} column was used Peaks appeared in the same place even though the Novopak was never exposed to albumin

13/ Finally a cleaning procedure supplied by Waters was tried This cleaning procedure was similar to the one above, but had an additional washing phase with chloroform Still no change in the elution profile was seen

Several runs without albumin injection were carried out and fractions were lyophilized No protein bands were observed in the albumin position in fractions 11 to 15 The bioactivity of these fractions were tested and the results are shown in Table 3 5 5 4 1

Table 3.5.5.4.1 Bioactivity of HPLC fractions

VARIABLES	DE/ ASSAY 1	DE/ ASSAY 2	CN/ ASSAY 1
1% DHS	100 0 ± 16 7	100 0 ± 16 6	100 0 ± 6 75
+ fraction 1	40 00 ± 6 67	44 44 ± 13 9	43 72 ± 4 17
+ fraction 2	36 66 ± 10 0	36 11 ± 11 1	41 99 ± 4 17
+ fraction 3	30 00 ± 6 67	25 00 ± 5 55	33 77 ± 0 00
+ fraction 4	36 66 ± 10 0	33 00 ± 11 1	35 50 ± 9 75
+ fraction 5	40 00 ± 13 3	33 00 ± 11 1	34 20 ± 0 75
+ fraction 6	26 66 ± 6 67	27 78 ± 8 33	24 00 ± 0 92
+ fraction 7	30 00 ± 6 67	30 55 ± 11 1	40 00 ± 4 04
+ fraction 8	23 33 ± 6 67	13 90 ± 8 33	25 71 ± 0 00
+ fraction 9	56 50 ± 8 70	52 02 ± 13 9	60 00 ± 6 06
+ fraction 10	52 20 ± 8 70	58 96 ± 10 4	61 43 ± 8 08
+ fraction 11	52 20 ± 13 0	83 24 ± 13 9	57 62 ± 12 5
+ fraction 12	56 50 ± 8 70	90 17 ± 17 3	79 52 ± 4 59
+ fraction 13	69 56 ± 17 4	104 0 ± 20 8	92 00 ± 13 1
+ fraction 14	73 90 ± 26 1	65 89 ± 13 9	52 00 ± 17 5
+ fraction 15	69 56 ± 17 4	65 90 ± 6 94	68 00 ± 14 1
5% DHS	443 5 ± 30 4	360 1 ± 39 7	440 7 ± 33 5

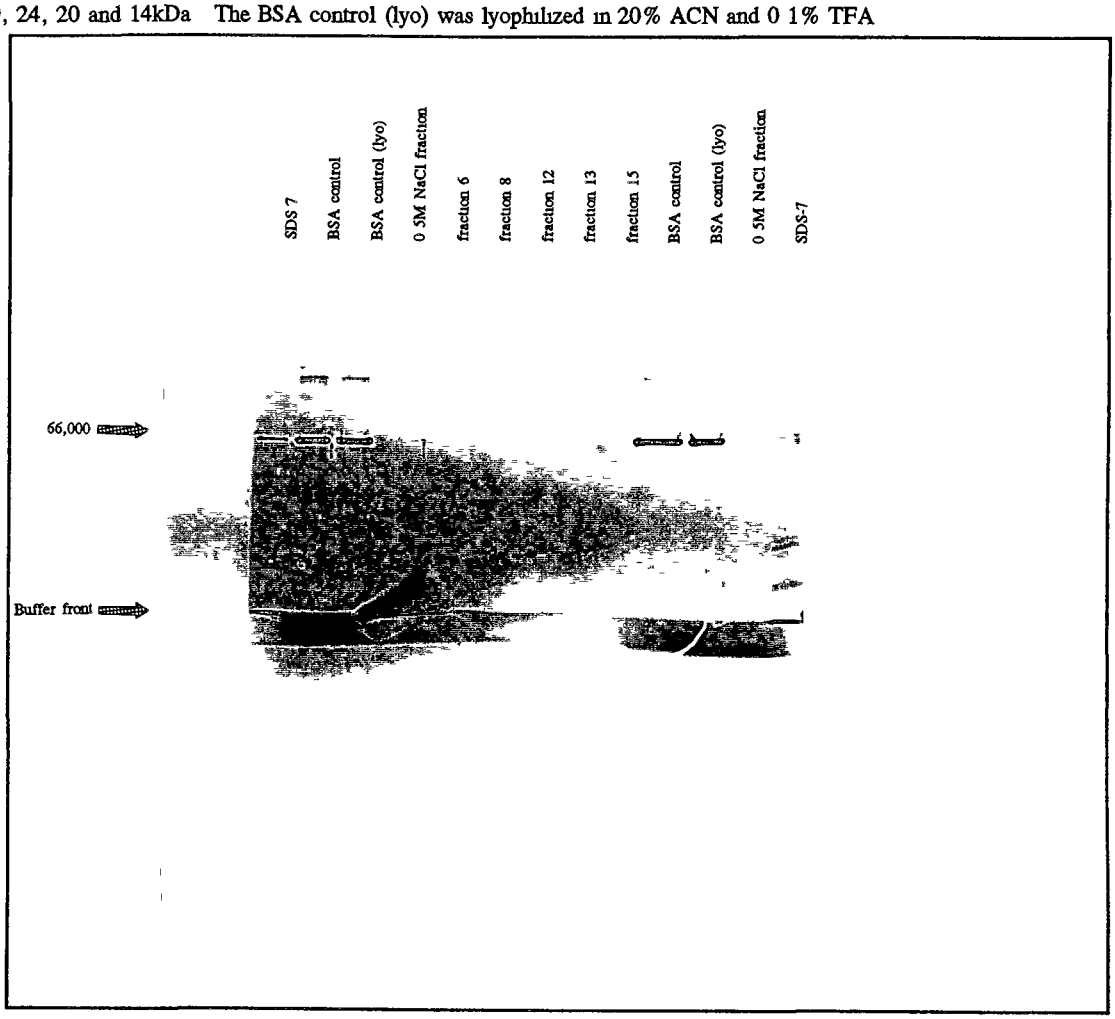
Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8 for DE and n=3 for CN) Abbreviations DE = dye elution, CN = cell number

The results were surprising in that most of the fractions showed growth inhibition in comparison to the control (1% DHS) It was as if some residual acetonitrile or TFA had not been fully

removed during lyophilization There was no visible residue left in any of the fractions In addition, there was no change in colour on addition of the basal medium that would be expected if some acetomtrile or TFA remained In the earlier fractions (1 - 8), inhibition of 60 - 70% was seen. For fractions 9 - 11, the inhibition was 40 - 50%, while the least inhibitory fractions occurred towards the end of the gradient with 13 being the least inhibitory

A gel was run with fractions 6, 8, 11, 12, 13, 14 and 15 to see if any residual albumin was eluting off at these conditions (Figure 3 5 5 4 2) The gel showed no protein bands at the albumin position, whereas when the albumin had been injected, protein migrating the same distance as the albumin in the SDS gels had been detected

Figure 3.5.5.4.2 Gel of HPLC fractions Samples were applied at 30μl per well with a concentration of 10ng/ml (see materials and methods, section 2 13 3) The SDS control shows a range of molecular weight markers 66, 45, 36, 29, 24, 20 and 14kDa The BSA control (lyo) was lyophilized in 20% ACN and 0 1% TFA



These results indicated that the peaks appearing in fractions 12 - 15 had no stimulatory ability However, the inhibition was worrying in that there was no inhibition in the first set of experiments Could it be, that some unfavourable reaction was taking place in the column and

rendering the fractions collected inhibitory? To see if the activity of the fractions had decreased, samples were run as before using the BSA The results are shown in Table 3 5 5 4 3

Table 3.5.5.4.3 Bioactivity of HPLC fractions after application of BSA

VARIABLES	CN/ ASSAY 1	DE/ ASSAY 1	DE/ ASSAY 2
1% DHS	100 0 ± 0 76	100 0 ± 10 7	100 0 ± 8 78
+ fraction 1	64 20 ± 9 14	51 78 ± 8 17	51 50 ± 3 47
+ fraction 2	68 45 ± 5 56	49 30 ± 7 86	49 20 ± 5 72
+ fraction 3	82 71 ± 19 8	58 30 ± 4 17	66 66 ± 11 4
+ fraction 4	64 53 ± 4 94	51 77 ± 9 23	47 73 ± 5 57
+ fraction 5	89 84 ± 1 51	55 21 ± 1 96	43 18 ± 5 25
+ fraction 6	99 82 ± 13 9	76 74 ± 11 1	61 93 ± 6 53
+ fraction 7	64 76 ± 1 32	11 23 ± 2 30	21 60 ± 6 01
+ fraction 8	13 21 ± 2 29	0 00 ± 0 00	14 20 ± 6 53
+ fraction 9	63 88 ± 17 8	66 09 ± 5 72	65 15 ± 3 91
+ fraction 10	69 16 ± 14 0	57 14 ± 6 73	65 15 ± 6 57
+ fraction 11	79 73 ± 5 50	66 09 ± 6 23	63 10 ± 7 99
+ fraction 12	100 4 ± 3 96	72 76 ± 11 6	88 38 ± 12 2
+ fraction 13	92 30 ± 6 15	93 99 ± 18 8	94 80 ± 12 3
+ fraction 14	100 9 ± 4 92	86 96 ± 19 2	78 80 ± 11 1
+ fraction 15	96 82 ± 8 94	94 91 ± 6 89	108 5 ± 11 2
5% DHS	223 6 ± 65 0	291 9 ± 22 8	317 0 ± 29 8

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8 for DE and n=3 for CN) Abbreviations CN = cell number, DE = dye elution

The biological activity of the fractions of albumin was tested again and showed inhibition in the range of fractions from 1 to 10, except 5 and 6, which had almost lost inhibition Fraction 8 which contained the bulk of the albumin applied to the column and showed greater than 90% inhibition For fractions 12 -15, there was no inhibition Yet the elution profiles were the same as before

The biological activity of the 0 5M NaCl fraction was tested There was only enough of the 0 5M NaCl fraction to carry out one experiment The results are shown in Table 3 5 5 4 4 For the 0 5M NaCl fraction, 25 - 45% inhibition was seen in fractions 1 - 7 Fractions 8, 9

and 10 which contain the bulk of the albumin showed 50 - 54% inhibition From fraction 11 onwards, the extent of inhibition was similar to that seen with fractions 1 - 7 except fraction 14 where 53% inhibition was seen

Table 3.5.5.4.4 Bioactivity of HPLC fractions after application of 0 5M NaCl fraction

VARIABLES	BSA	0 5M NaCl	NO INJECTION
1% DHS	100 0 ± 0 76	100 0 ± 8 33	100 0 ± 6 75
+ fraction 1	64 20 ± 9 14	64 49 ± 13 6	43 72 ± 4 17
+ fraction 2	68 45 ± 5 56	57 55 ± 4 89	41 99 ± 4 17
+ fraction 3	82 71 ± 19 8	75 92 ± 1 73	33 77 ± 0 00
+ fraction 4	64 53 ± 4 94	58 16 ± 6 86	35 50 ± 9 75
+ fraction 5	89 84 ± 1 51	76 30 ± 5 53	34 20 ± 0 75
+ fraction 6	99 82 ± 13 9	63 06 ± 4 33	24 00 ± 0 92
+ fraction 7	64 76 ± 1 32	62 60 ± 10 4	40 00 ± 4 04
+ fraction 8	13 21 ± 2 29	46 95 ± 7 15	25 71 ± 0 00
+ fraction 9	63 88 ± 17 8	48 47 ± 6 51	60 00 ± 6 06
+ fraction 10	69 16 ± 14 0	50 38 ± 4 99	61 43 ± 8 08
+ fraction 11	79 73 ± 5 50	67 56 ± 22 9	57 62 ± 12 5
+ fraction 12	100 4 ± 3 96	75 95 ± 14 3	79 52 ± 4 59
+ fraction 13	92 30 ± 6 15	62 90 ± 14 9	92 00 ± 13 1
+ fraction 14	100 9 ± 4 92	47 52 ± 2 43	52 00 ± 17 5
+ fraction 15	96 82 ± 8 94	65 90 ± 6 12	68 00 ± 14 1
5% DHS	223 6 ± 28 9	299 2 ± 13 4	440 7 ± 33 5

Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=3) All results were determined by cell number

The extent of inhibition was very similar in fractions 1 - 4 (both showed a slight loss of inhibition at fraction 3, which corresponded to the position where the coproporphyrin was eluted off) For fraction 5, there was a loss of inhibition Fraction 6 showed diverging results for the BSA runs there was an almost total loss of inhibition while this was not seen with the 0 5M NaCl sample (37% inhibition)

At fractions 8 - 9, where the BSA was most strongly inhibitory (90 - 100%), the extent of inhibition with the 0 5M NaCl was only 54 - 58% inhibitory In the rest of the fractions, discrepancies existed from 12 through to 15 At 12 and 15, there was more inhibition seen with the 0 5M NaCl sample than with the native BSA, the extent of inhibition corresponded to that

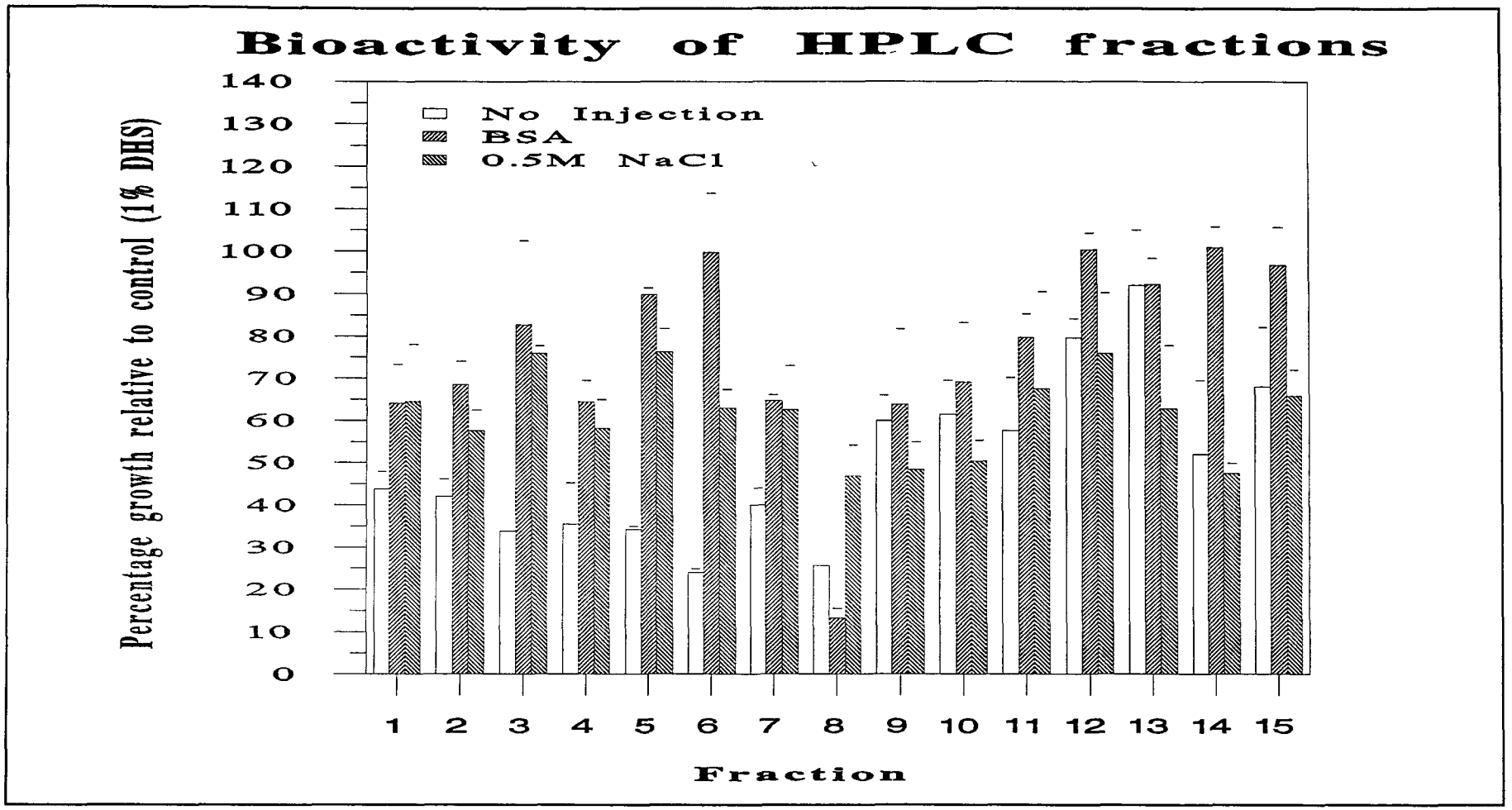


Figure 3.5.5.4.4 Growth response of NRK cells to rp-HPLC fractions in low serum medium Results are expressed as the average percentage growth relative to control (1% DHS) $\pm$ standard deviation (n=3) Cell number was used as the end point of two separate assays

seen when no injection was made which could suggest something on BSA that was slightly stimulatory was lost on isolation of the 0 5M NaCl heparin sepharose fraction At fraction 13, the 0 5M NaCl sample was more inhibitory than the BSA or the control (no injection) run At 14, the 0 5M NaCl fraction showed the same extent of inhibition seen in the control but not seen with fraction 14 from the BSA runs

The fact that inhibition was seen in the control, BSA and 0 5M NaCl fraction and not seen before, lends little weight to the results for biological activity

3.5.5.5 Comparison of elution profiles

To see if any gross variations existed in the elution profiles of the BSA used here and the acidic BSA used by Congote (Sigma A8022) The elution profiles were compared at two sensitivity ranges (0 - 1 0Aufs and 0 - 0 2Aufs) In addition, BSA fatty acid free and the 0 5M NaCl fraction were also compared

3 5 5 5 1 BSA (A4919)

At a sensitivity range of 0 - 1 0Aufs, there was a small blip at 12 -17mm as in the first set of experiments The main peak was at 70 - 90mm The three small peaks appeared towards the end of the gradient At a more sensitive detection level of 0 - 0 2Aufs (Figure 3 5 5 5 1), the small peak in front of the main protein peak was symmetrical and clearly distinct from the main peak The three peaks at the end of the gradient were seen as before

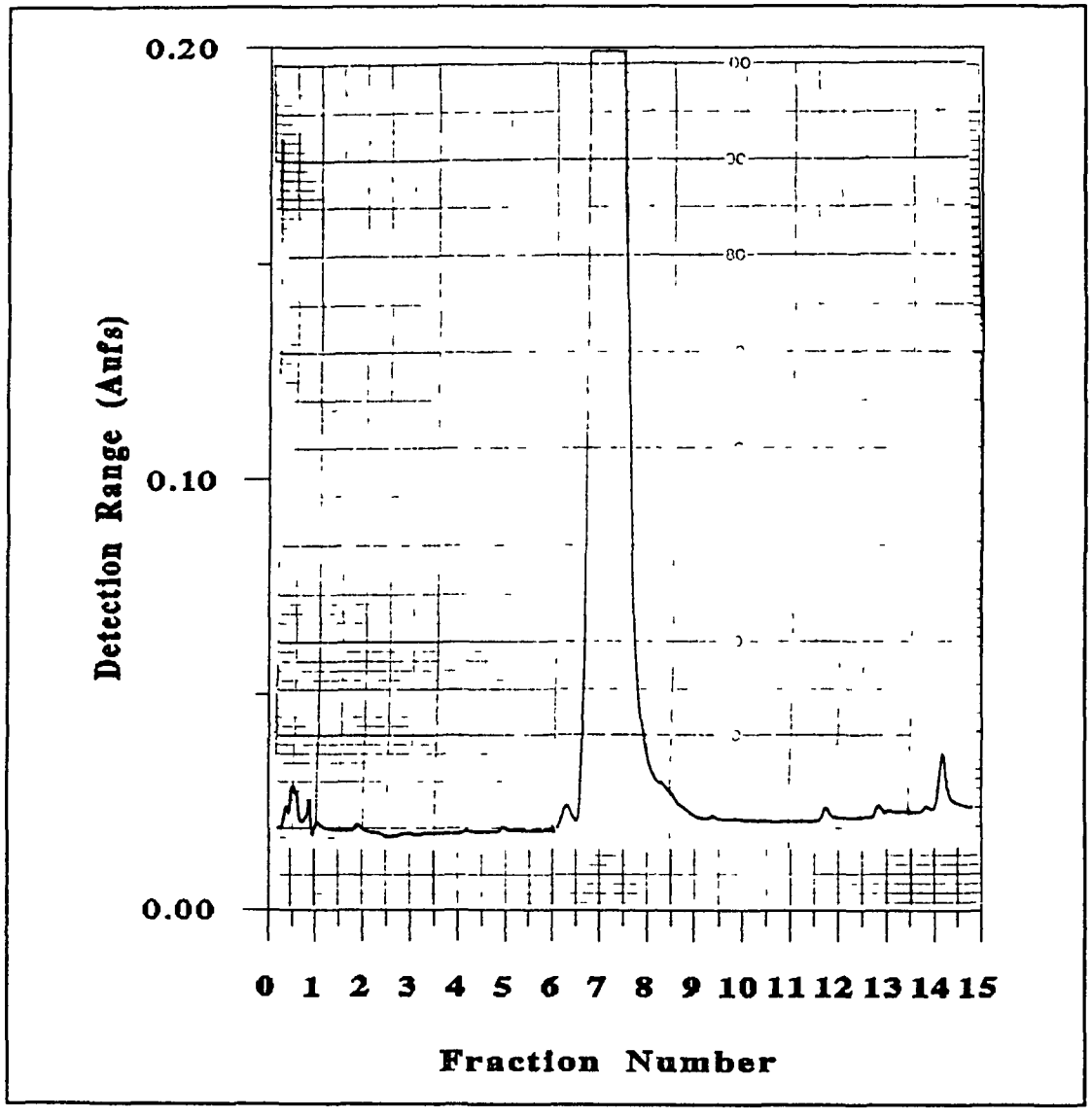


Figure 3.5.5.5.1 Elution profile for 100mg/ml BSA (A4919) on a C18 μBondapak column The absorbance was detected at 280nm The chart speed was set at 5mm/min with a detection range of 0 - 0 2 Aufs

3.5.5.5.2 Acidic BSA (A8022)

At low sensitivity range (0 - 2 0Aufs), the acidic albumin showed the same elution profile as the BSA used for all the previous experiments (Figure 3 5 5 5 2) At a more sensitive range (0 0 - 0 2Aufs), the same trend existed except an additional blip was seen about 20mm The small peak immediately in front of the main protein peak was present but was not as high or as symmetrical as the peaks seen for BSA (A4919) or BSA-FAF

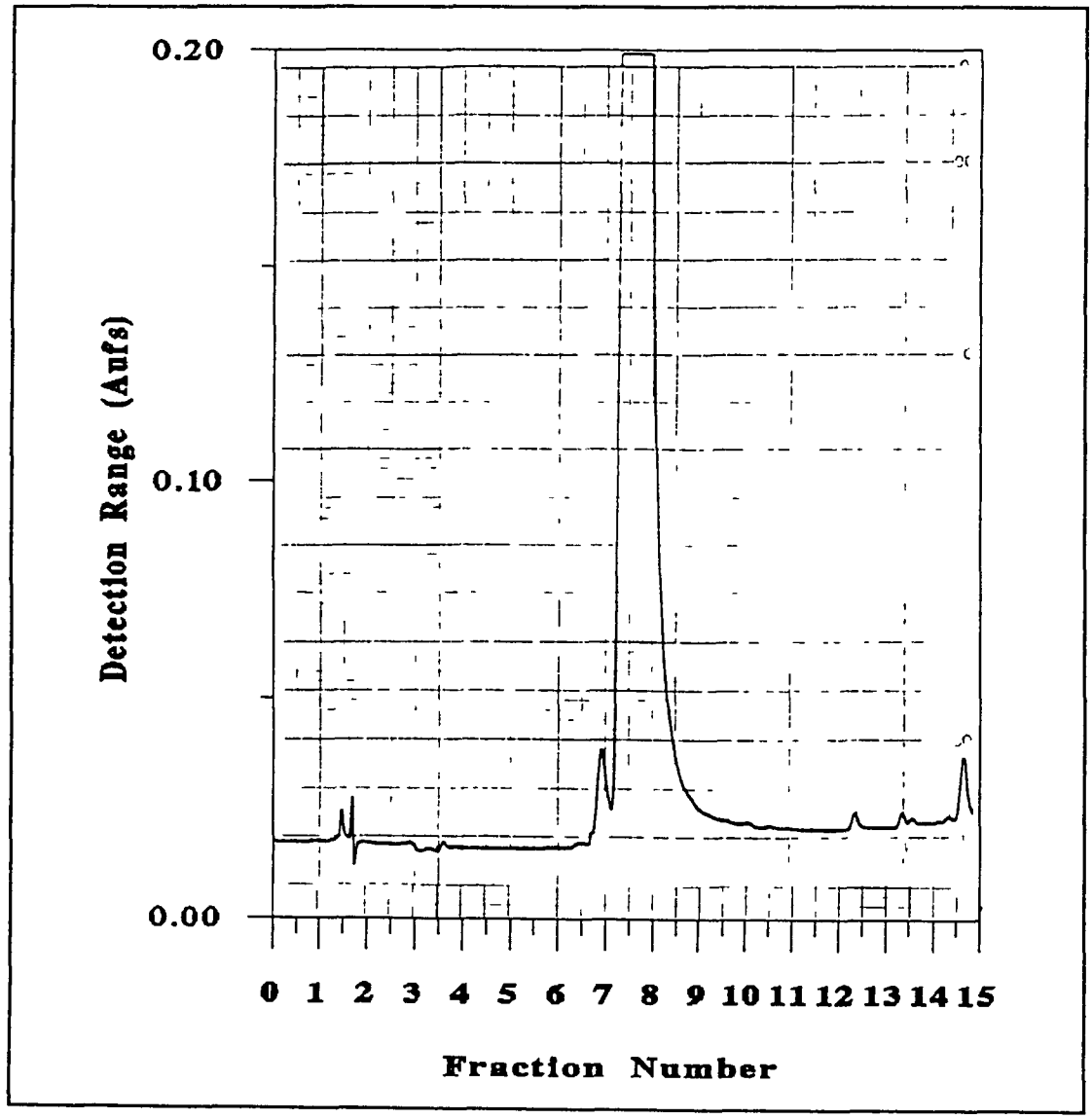


Figure 3.5.5.5.2 Elution profile for 100mg/ml acidic BSA (A8022) on C18 μBondapak column The absorbance was detected at 280nm The chart speed was 5mm/min with a detection range of 0 - 0 2Aufs

3.5.5.5.3 BSA-faf

BSA fatty acid free showed the same elution profile as the other two albumins (Figure 3 5 5 5 3) at the lower sensitivity range (0 - 2 0Aufs) At 0 - 0 2Aufs, the blip at 15mm was seen to consist of a peak and trough The small peak immediately before the main protein peak was isolated from the main peak and was symmetrical in shape Again the three small peaks towards the end of the gradient were present

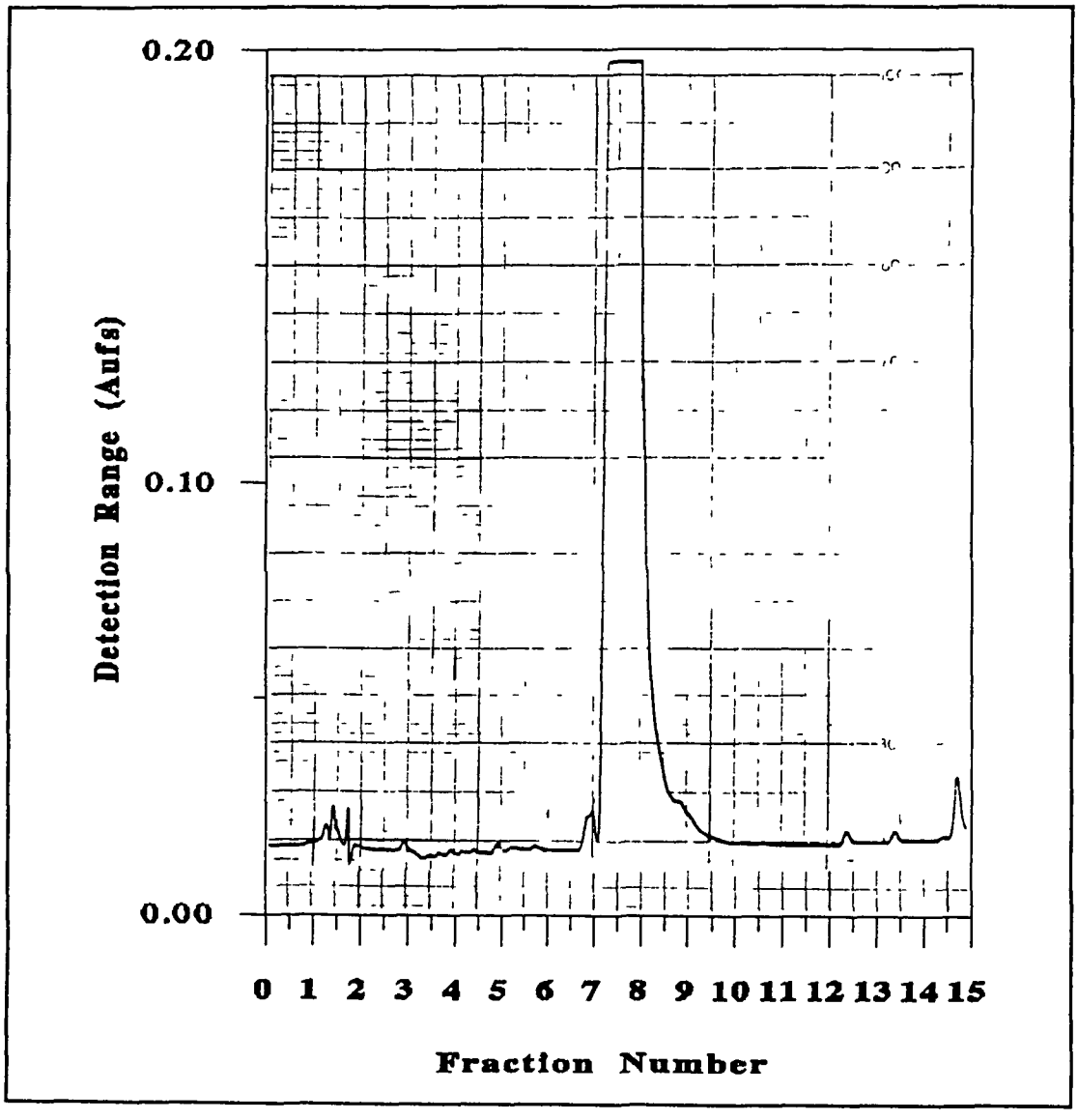


Figure 3.5.5.5.3 Elution profile for 100mg/ml fatty acid free - BSA(A6003) on C18 μBondapak column. The absorbance was detected at 280nm The chart speed was 5mm/min with a detection range of 0 - 0 2Aufs

3.5.5.5.4 0.5M NaCl fraction

At 0 - 1 0Aufs, the elution profile of the 0 5M NaCl fraction was similar to that of the other albumins, except the size of the main protein peak was much smaller (Figure 3 5 5 4) At a more sensitive range (0 - 0 2Aufs), the blip at 15mm consisted of a peak and trough An additional blip was seen at 50mm At 80mm, the main protein peak showed a sharp start (without a lip or smaller peak) and the tailing effect The three small peaks were present between 120 and 150mm No concentration of a specific protein other than at the albumin position was seen

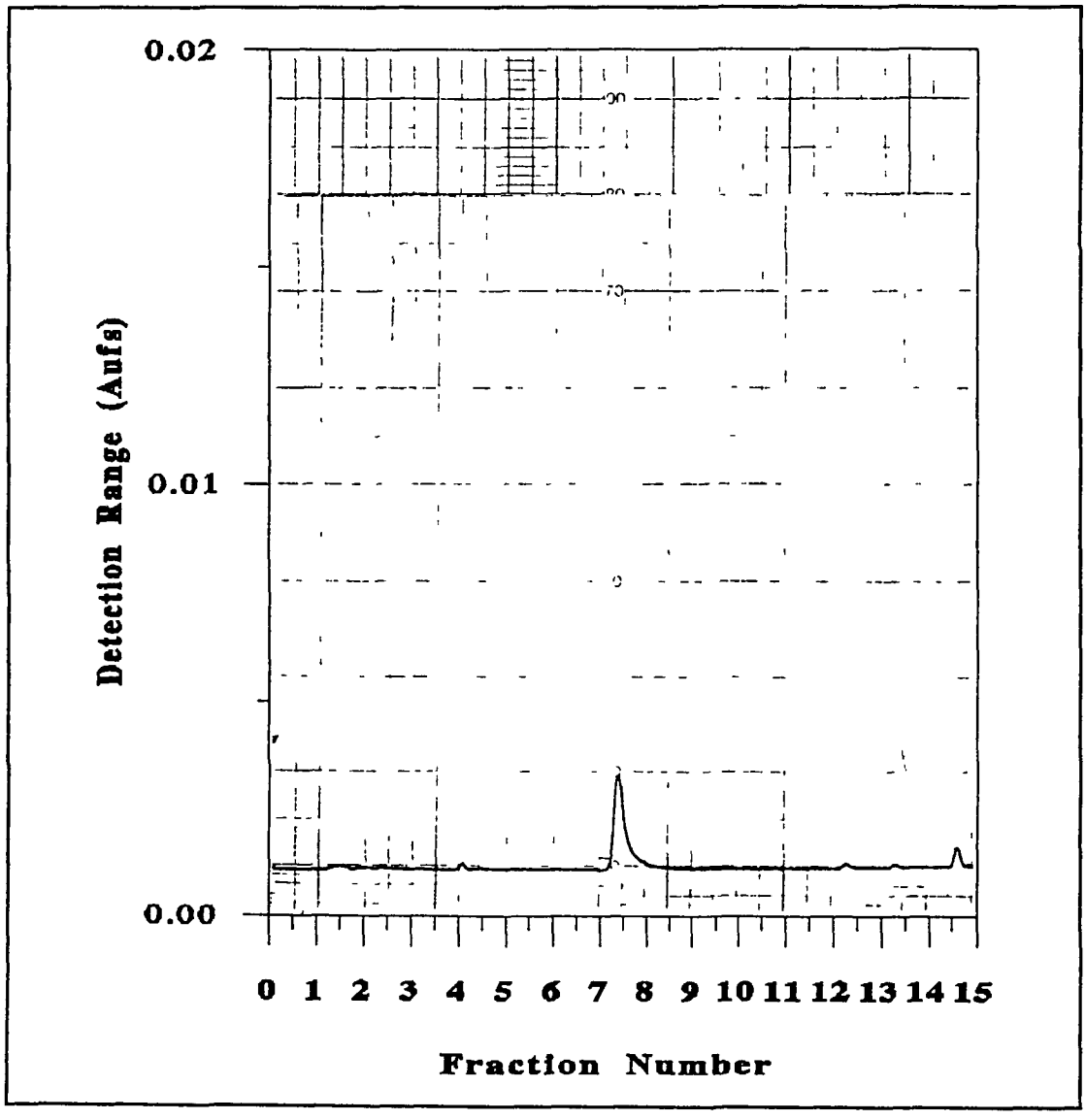


Figure 3.5.5.5.4 Elution profile for the 0 5M NaCl fraction on C18 μBondapak column The absorbance was detected at 280nm The chart speed was set at 5mm/min with a detection range of 0 - 0 02 Aufs

In addition, coproporhyrin was run again alone (Figure 3.5.5.5.5). A peak and trough were seen at 15mm with a small asymmetrical bump at 80mm. When a control (no injection) was run immediately after these samples (Figure 3.5.5.5.6), the presence of a small peak at 80mm indicated the presence of residual protein on the column. The three peaks at the end of the gradient were there as before.

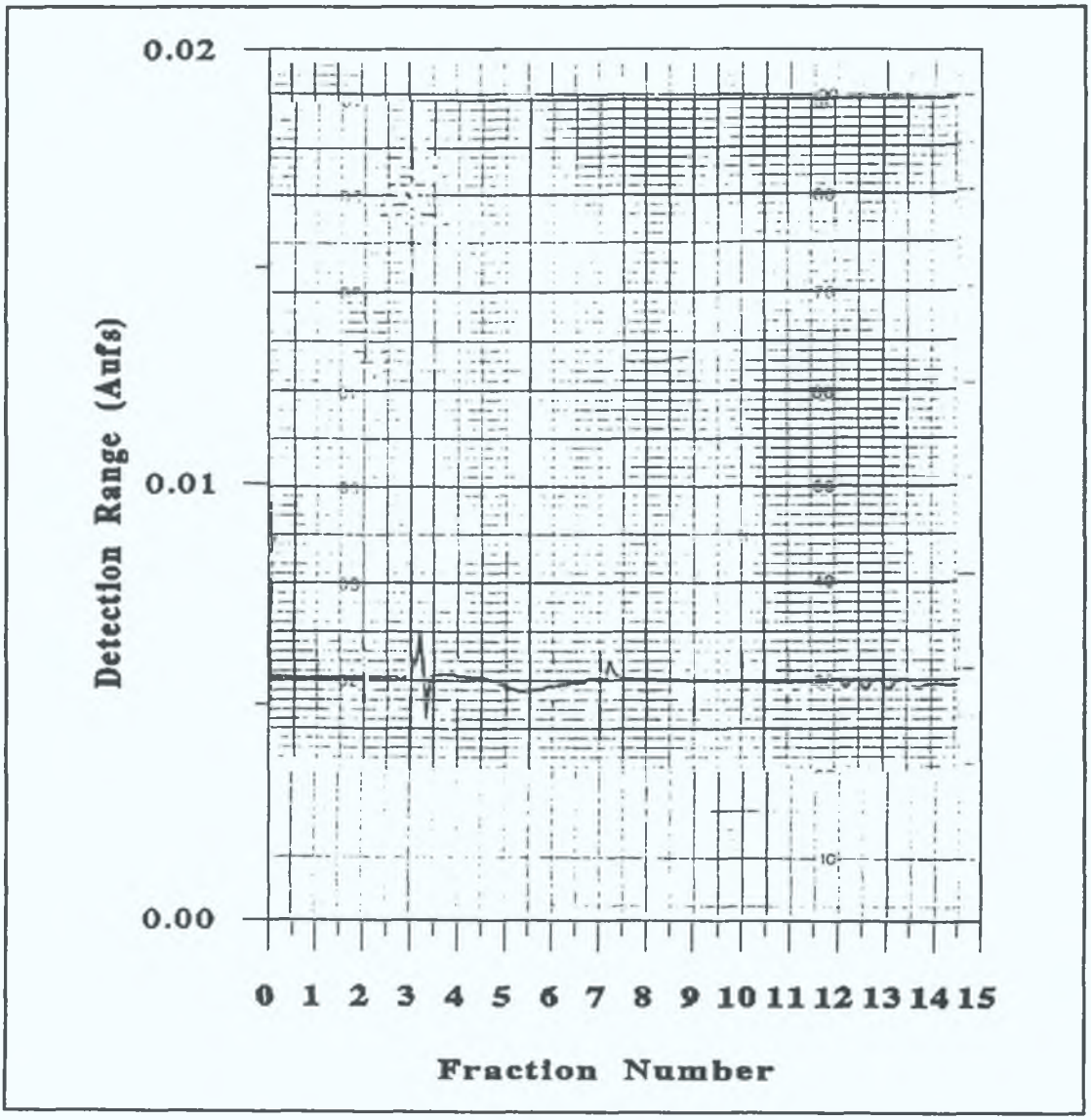


Figure 3.5.5.5.5 Elution profile for 500μg/ml Coproporphyrin on C18 μBondapak column. The absorbance was detected at 400nm. The chart speed was set at 5mm/min with a detection range of 0 - 0.02 Aufs.

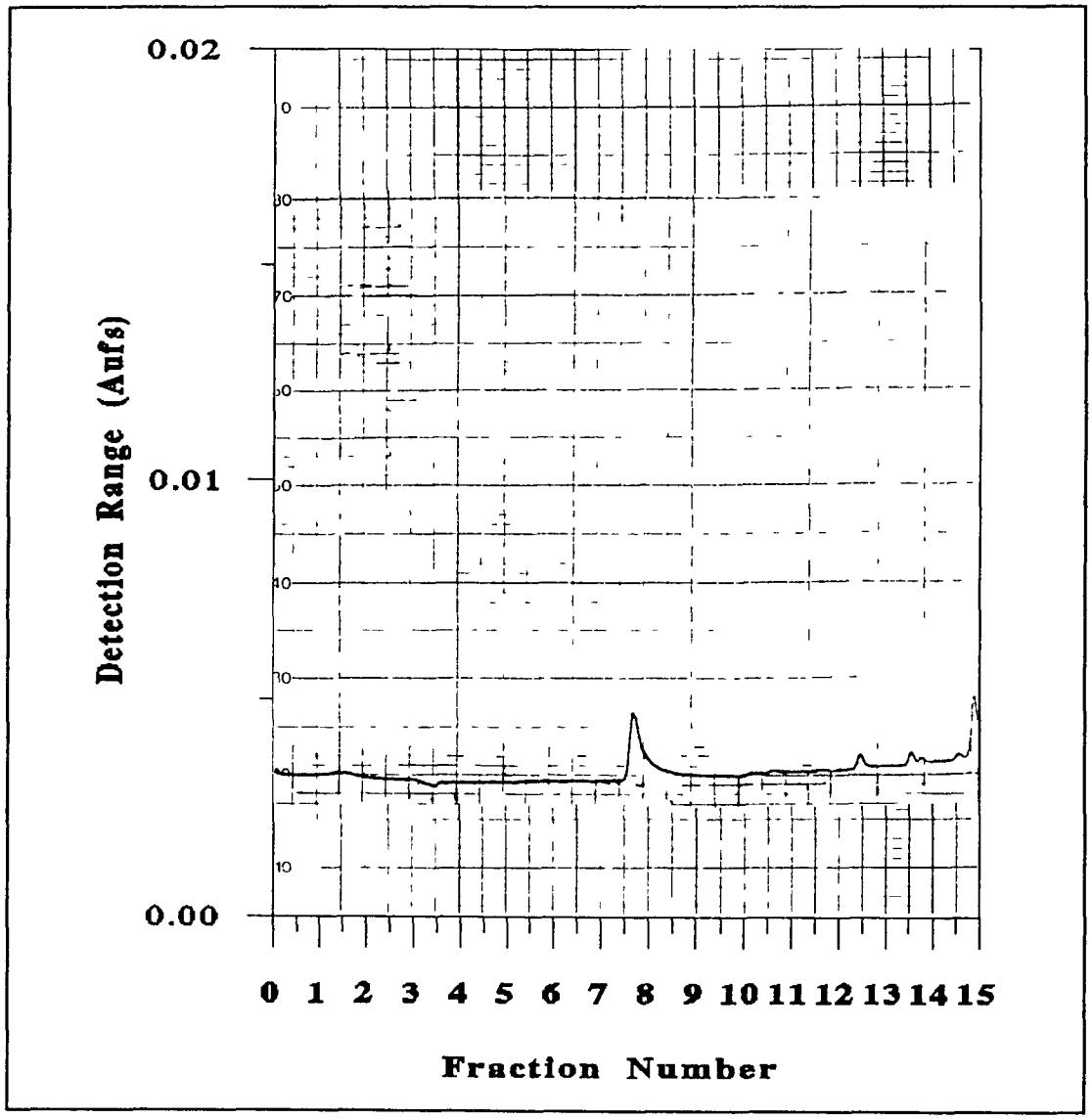


Figure 3.5.5.5.6 Elution profile for the control (no albumin injection) on C18 μBondapak column The absorbance was detected at 280nm The chart speed was set at 5mm/min with a detection range of 0 - 0 02 Aufs

In conclusion, reverse phase HPLC was not a successful means of trying to isolate the activity from BSA based on the procedure used by Congote (1987) For the first experiment, activity was seen in two fractions (fraction 6 and 12) Neither coincided with the fraction in which the bulk of protein or coproporphyrin were eluted from the column, while inhibition was seen with the bulk of albumin The second set of experiments were run because of the presence of three persistent small peaks between fractions 12 and 15 even when no albumin was being injected onto the column The control run (without albumin injection), the BSA and the 0 5M NaCl fraction were run and all fractions obtained showed varying degrees of inhibition Due to the variability in the biological response of the NRK cells, no more separations were tested on the cells

The elution profile for the BSA was different to that obtained by Congote It was not due to differences in the albumin used, as acidic BSA (A8022), the albumin used by Congote showed the same profile as the BSA used here (Figure 3 5 5 5 1 and 2)
The size of the column and the alterations in the programme could also have affected the elution profile Care was taken to ensure that the column (half the diameter of Congote's and therefore a quarter of the volume) was not overloaded with albumin

3.5.6 GEL FILTRATION

Gel filtration was used to try and determine the molecular weight of the active fraction from the 0 5M NaCl heparin sepharose wash It was of interest to see if the activity was eluted off in the same region as albumin or if the activity that eluted off in the 0 5M NaCl fraction could be related to Congotes' erythrotropin-like factor, which has an apparent molecular weight of about 9000

3.5.6.1 Calibration of column

An S200 column was set up as described in section 2 20 To calibrate the column a number of molecular weight markers ranging from 12,000 to 200,000 were run through the column The elution volume (Ve) of each of the molecular weight markers was used to calculate Ve/Vo and plotted against the log of the molecular weight (Figure 3 5 **6** 1)

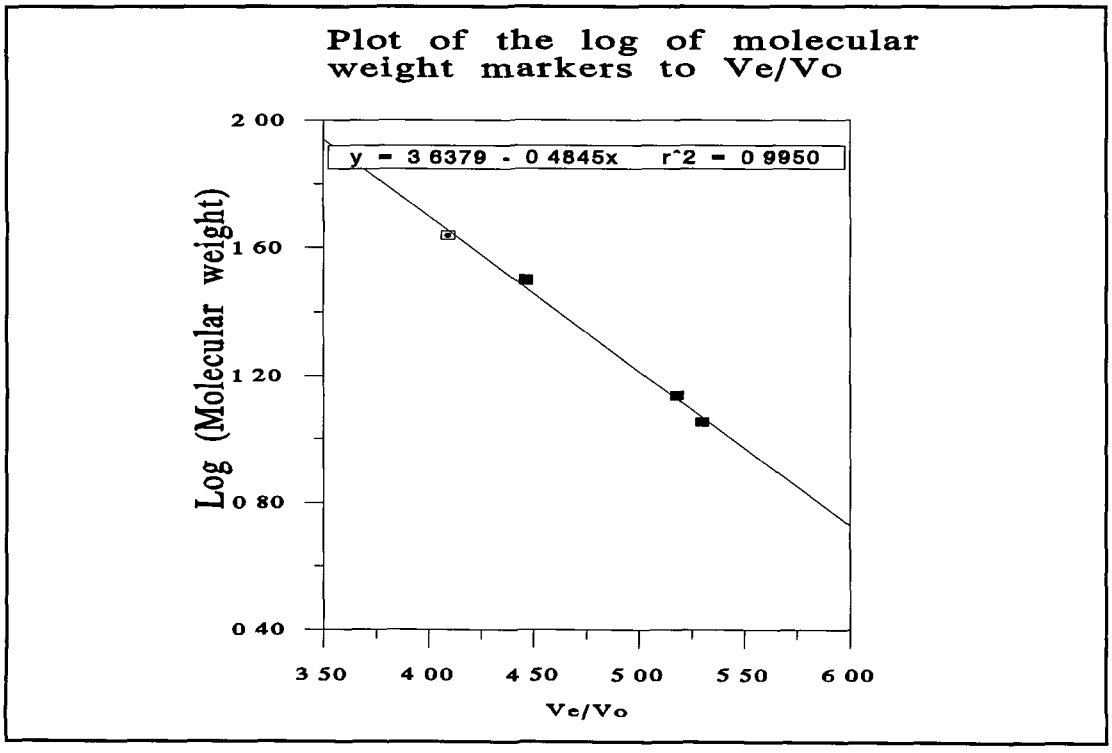


Figure 3.5.6.1 Plot of Ve/Vo of molecular weight (Mw) markers against the log of the molecular weight The void volume (Vo) from run 1 on the S200 column was found by determining the fraction at which Blue dextran (Mw = 200,000) gave maximal absorbance (fraction 36) The standards used were ß-Amylase (Mw 200,000), Alcohol dehydrogenase (Mw 150,000), Carbonic anhydrase (Mw 29,000) and Cytochrome C (Mw 12,400) BSA has a molecular weight of about 66,000 and so by extrapolating from the graph should appear in fractions 46 - 48

3.5.6.2 Biological activity of fractionated 0.5M NaCl

2 5ml of 0 5M NaCl fraction (equivalent to 25mg/ml albumin) was applied to the column The buffer used to wash the sample through the column was phenol red-free DME Phenol red-free DME was used so that the phenol red colour which could bind to BSA, would not interfere with the absorbance at 280nm It also meant that fractions did not have to be diafiltered into a basal medium to assay bioactivity As approximately, a 1 in 5 dilution occurred as the sample passed through the column, the concentration in the fractions would be about 10mg/ml No dilution was required to assay the fractions in the low serum-supplemented medium (1% DHS) As the gel filtration was carried out using DME phenol red-free as the buffer, cells were prepared in Ham's F12 with serum, to give a final concentration equivalent to 5mg/ml BSA in ATCC For the serum-free assays, the final concentration was diluted down to 0 1mg/ml Two separate assays were set up with a low serum background (1% DHS) and two serum-free assays were set up, each with different serum-free media The simple SFM (SFMs) composed of 10μg/ml insulin and 1 39μg/ml Fe_2SO_4 in McCoys 5a medium The complex SFM (SFMc) in addition contained 1ng/ml ß-FGF and 5ng/ml PDGF

The results for two separate assays in the low serum medium (1% DHS) are shown in Figure 3 5 6 2 1 and Table 3 5 6 2 Fractions 31 to 86 were assayed for biological activity Slight stimulation was seen in fractions 31 to 35 Beyond fraction 36, the activity became inhibitory reaching a maximum of 60 - 67% inhibition relative to the control (1% DHS) in fractions 41 - 42 Thereafter, a gradual reduction in inhibition occurred until fractions 52 - 53 After this, the fractions became stimulatory again reaching a maximum of 60% above the control (1% DHS) in fraction 60 to 63 with a sudden loss of stimulation in fraction 64 No growth occurred in fraction 64 The total loss of growth may have been due to not inoculating the wells with cells If some inhibitory factor prevented the cells from attaching and growing, the effect should have been seen in the serum-free medium, where the cells have much less protection against inhibitory factors

In fraction 66, there was a slight drop in stimulation with further increases of up to 50% growth above the control in fractions 68 - 70 Up until fraction 70, duplicate assays showed similar results However, in fraction 71, a 3-fold stimulation was seen in assay 2 while only 50% stimulation above the control was seen in assay 1 Thereafter, both assays showed a reduction in stimulation (to about 20 - 30% above the control) with a small increase of 50% relative to the control in fraction 84 The 0 5M NaCl control (from Run 10, Appendix G) was only tested in the serum-free assays as there was not enough to assay in the low serum assays

Table 3.5.6.2 Biological activity of fractions from Gel filtration 1 of 0 5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	SFMs	SFMc	A_{280nm}
Control	100 0 ± 5 46	100 0 ± 5 29	100 0 ± 3 83	100 0 ± 6 42	0 000
31	134 8 ± 9 58	139 4 ± 6 78	103 7 ± 5 38	108 5 ± 6 52	-0 011
32	90 20 ± 7 55	105 2 ± 7 54	97 20 ± 4 09	116 3 ± 12 1	0 004
33	125 9 ± 7 94	123 9 ± 6 06	97 33 ± 5 62	116 8 ± 12 0	-0 001
34	121 1 ± 3 37	137 7 ± 7 69	96 66 ± 6 71	106 5 ± 4 71	0 005
35	123 4 ± 8 61	126 3 ± 8 42	94 90 ± 0 10	110 6 ± 1 21	0 029
36	92 84 ± 4 18	85 24 ± 4 05	95 49 ± 3 00	111 1 ± 6 67	0 115
37	60 27 ± 3 59	58 33 ± 3 54	99 67 ± 4 95	125 7 ± 9 92	0 094
38	49 05 ± 2 96	47 43 ± 2 38	106 8 ± 4 75	115 4 ± 7 46	0 081
39	46 17 ± 3 71	46 24 ± 2 97	100 3 ± 6 00	134 2 ± 9 36	0 087
40	48 23 ± 3 41	46 66 ± 2 36	107 7 ± 6 82	115 8 ± 1 85	0 078
41	39 33 ± 4 33	32 24 ± 2 26	104 9 ± 3 63	101 6 ± 4 53	0 063
42	33 22 ± 8 99	34 30 ± 8 02	105 2 ± 0 01	100 8 ± 6 53	0 048
43	57 88 ± 8 99	50 37 ± 6 32	103 1 ± 6 74	104 6 ± 4 04	0 039
44	57 25 ± 4 42	55 89 ± 4 66	95 20 ± 4 64	103 7 ± 7 66	0 028
45	59 20 ± 2 91	48 41 ± 4 33	92 57 ± 10 5	103 3 ± 6 27	0 030
46	56 30 ± 3 17	39 06 ± 4 12	113 5 ± 6 92	100 8 ± 3 28	0 021
47	63 11 ± 5 70	48 66 ± 5 22	121 1 ± 14 1	108 5 ± 3 48	0 047
48	71 24 ± 5 03	53 90 ± 3 49	131 4 ± 13 6	103 2 ± 6 28	0 009
49	83 30 ± 9 81	71 00 ± 5 78	99 76 ± 9 56	103 9 ± 6 97	0 002
50	76 40 ± 2 57	66 10 ± 8 37	97 11 ± 3 47	114 4 ± 9 38	0 001
51	74 53 ± 3 76	74 27 ± 8 87	100 9 ± 11 6	103 2 ± 3 60	0 005
52	86 28 ± 8 14	103 3 ± 8 59	84 37 ± 7 12	106 0 ± 9 27	0 024
53	104 9 ± 10 1	123 6 ± 9 79	72 43 ± 5 47	98 56 ± 5 37	0 019
54	112 2 ± 9 56	138 9 ± 11 6	84 85 ± 11 2	104 2 ± 11 1	-0 005
55	100 9 ± 7 07	123 9 ± 10 2	84 61 ± 9 54	101 6 ± 6 27	0 004
56	128 9 ± 9 15	149 7 ± 6 21	76 30 ± 4 50	98 56 ± 9 76	-0 005
57	155 0 ± 15 6	141 8 ± 7 50	75 00 ± 6 89	80 45 ± 4 57	-0 007
58	160 9 ± 12 8	146 9 ± 8 41	96 20 ± 2 14	108 1 ± 8 89	-0 000
59	151 9 ± 11 1	150 4 ± 7 93	106 9 ± 12 5	105 8 ± 7 90	0 022
60	174 6 ± 12 2	154 9 ± 6 31	97 62 ± 6 53	97 70 ± 11 7	-0 011

Results are expressed as the average percentage growth relative to control (1% DHS for low sera assays (LS), SFMs and SFMc only for serum-free assays) ± standard deviation (n = 5 for low serum assays (DE as end point) and n = 4 (AP as end point) for serum-free assays) The absorbance was read at 280nm against DME phenol red-free medium

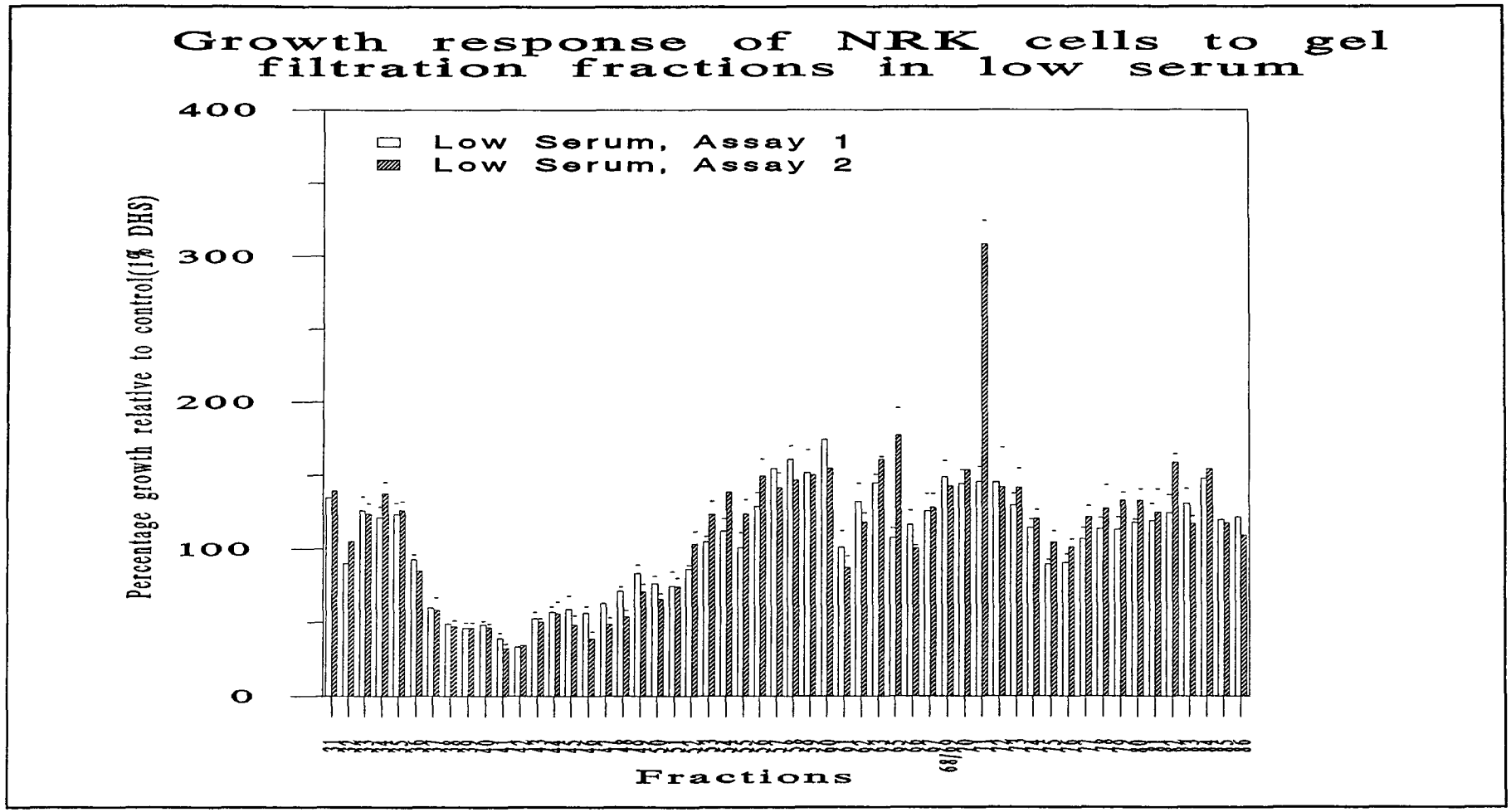


Figure 3.5.6 2.1 Growth response of NRK cells to gel filtration fractions of the 0 5M NaCl fraction in low serum medium Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=5) Dye elution was used as the end point for two separate assays

Table 3.5.6.2continued Biological activity of fractions from Gel filtration 1 of 0 5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	SFMs	SFMc	A_{280nm}
Control	100 0 ± 7 96	100 0 ± 3 03	100 0 ± 3 83	100 0 ± 11 7	0 000
61	101 2 ± 5 86	87 57 ± 1 79	95 31 ± 5 38	96 67 ± 6 50	0 007
62	132 4 ± 6 40	118 4 ± 18 5	90 49 ± 4 09	99 66 ± 3 50	0 017
63	145 5 ± 9 91	161 0 ± 2 09	95 13 ± 5 62	97 93 ± 8 46	0 031
64	0 000 ± 0 00	0 000 ± 0 00	99 41 ± 6 71	94 95 ± 1 99	0 059
65	107 8 ± 12 3	177 6 ± 9 64	97 30 ± 0 10	98 74 ± 8 26	-0 000
66	116 5 ± 10 8	100 5 ± 7 74	102 2 ± 3 00	116 9 ± 4 07	0 005
67	125 6 ± 9 63	128 1 ± 25 4	100 6 ± 4 95	86 08 ± 6 11	0 005
68/69	149 1 ± 7 03	142 8 ± 11 5	95 49 ± 4 75	89 37 ± 5 63	0 011
70	144 2 ± 9 97	153 7 ± 16 1	93 01 ± 6 00	81 60 ± 4 92	0 013
71	145 9 ± 11 2	308 3 ± 26 6	90 33 ± 6 82	90 80 ± 4 00	0 051
72	145 3 ± 8 08	142 2 ± 13 4	97 30 ± 3 63	84 39 ± 1 67	0 023
73	129 6 ± 6 18	141 5 ± 6 15	95 96 ± 0 01	82 92 ± 1 28	0 016
74	114 1 ± 3 21	120 4 ± 7 49	95 34 ± 6 74	83 94 ± 5 54	0 031
75	89 51 ± 5 59	104 6 ± 5 58	89 44 ± 4 64	100 3 ± 2 14	0 018
76	90 80 ± 7 42	101 2 ± 7 44	91 30 ± 10 5	99 09 ± 9 79	0 018
77	106 8 ± 7 46	121 9 ± 15 9	87 16 ± 6 92	96 71 ± 9 72	0 034
78	113 6 ± 8 51	127 4 ± 5 43	101 1 ± 14 1	88 50 ± 5 49	0 009
79	113 1 ± 2 24	133 0 ± 7 66	180 7 ± 13 6	84 76 ± 12 3	0 021
80	117 9 ± 11 7	133 0 ± 15 7	256 6 ± 9 56	97 37 ± 9 87	0 013
81	119 2 ± 12 3	125 0 ± 5 90	148 1 ± 3 47	114 9 ± 6 17	0 013
82	124 5 ± 10 2	158 7 ± 5 2	67 90 ± 11 6	82 37 ± 5 92	0 012
83	130 8 ± 9 71	117 4 ± 15 2	60 00 ± 7 12	80 82 ± 3 93	-0 007
84	147 8 ± 20 4	154 4 ± 5 37	71 11 ± 5 47	85 60 ± 8 87	-0 001
85	119 7 ± 8 52	117 7 ± 11 5	339 5 ± 11 2	92 94 ± 10 6	-0 009
86	121 6 ± 16 7	109 1 ± 6 17	------	------	-0 033
0 5Mc	------	-----	455 2 ± 73 8	160 8 ± 10 6	---

Results are expressed as the average percentage growth relative to control (1% DHS for assays 1 and 2, SFMs and SFMc only for serum-free assays) ± standard deviation (n = 5 for low serum assays and n = 4 for serum-free assays) The absorbance was read at 280nm against DME phenol red-free medium Dye elution was used as the end point for assays carried out in low serum-supplemented medium and acid phosphatase was used as the end point for assays carried out in SFM Abbreviations LS = low serum, 0 5Mc = 0 5M NaCl control

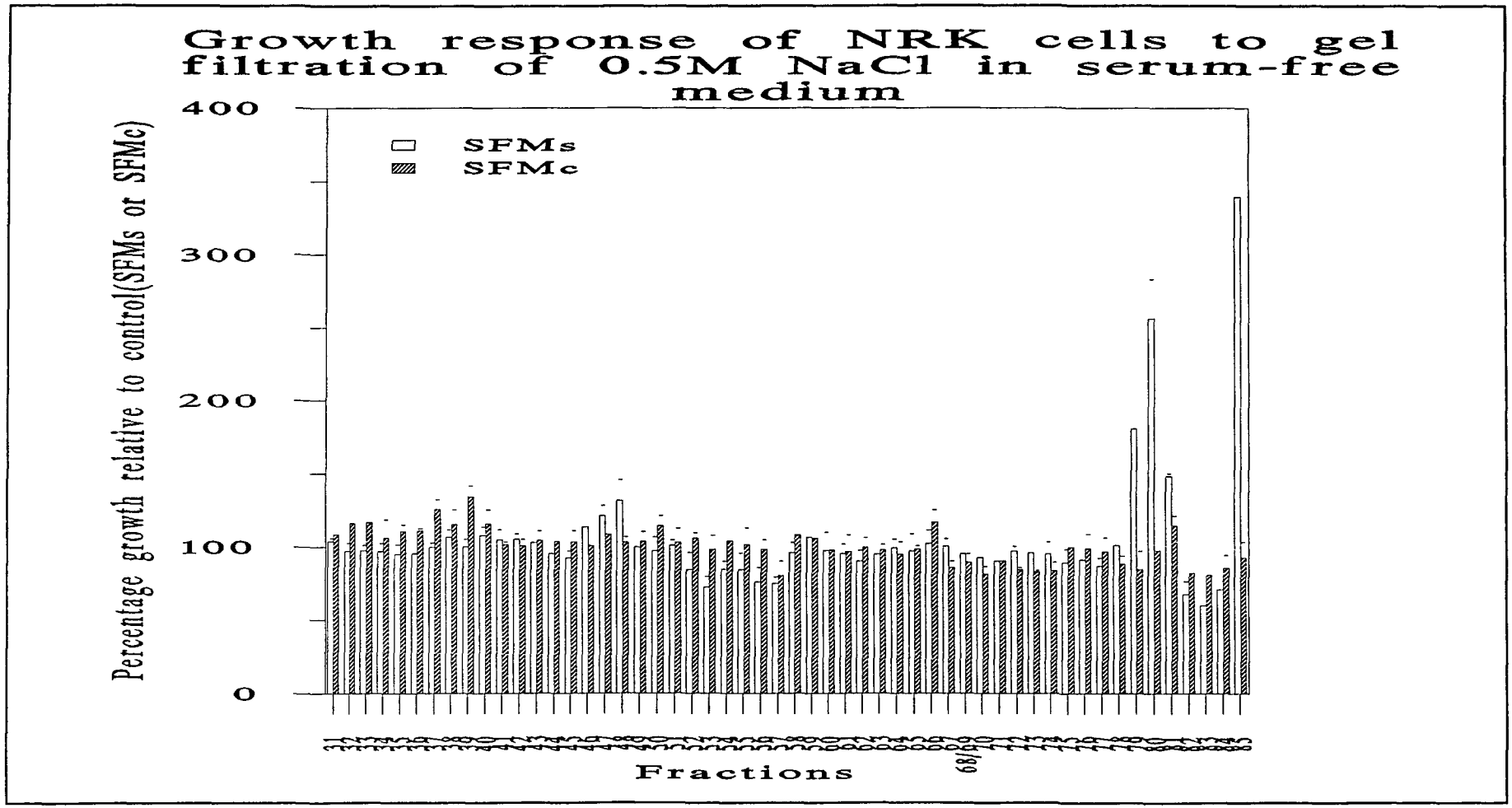


Figure 3 5 6.2.2 Growth response of NRK cells to gel filtration fractions of the 0 5M NaCl fraction in serum-free medium Results are expressed as the percentage growth relative to control (SFMs or SFMc) $\pm$ standard deviation (n=4) Acid phosphatase was used as the end point for assays

The results for the biological activity of the gel filtration fractions in SFM are shown in Figure 3 5 6 2 2 With the simple SFM (SFMs), very little stimulation or inhibition was seen until fractions 47 - 48, which showed a maximum of 30% stimulation above the control (SFMs) Slight stimulation was seen in fractions 53 and 57 of about 25% relative to the control Fraction 80 showed a 2 5-fold stimulation followed by 40% inhibition in fraction 83, with another peak stimulation of 3 4-fold in fraction 85 (molecular weight lower than 1,000) The control for the 0 5M NaCl fraction showed a 4 5-fold stimulation over the control
For the more complex SFM (SFMc), some stimulation was seen in fraction 39 (34% relative to control) Inhibition of 20% was seen in fraction 57 Except for fractions 39 and 57, little stimulation or inhibition was seen The 0 5M NaCl control showed 60% stimulation above the control (SFMc only)

The protein profile of the 0 5M NaCl fractionation showed a number of peaks at 280nm absorbance (Table 3 5 6 2 4) The first and largest occurred in fraction 36 To have gone straight through the column, it must have been a large weight molecule of 200,000 or greater (exclusion molecular weight of Dextran Blue) The second peak was seen in fraction 47, which corresponded to the theoretical position at which albumin should have eluted from the column This was only the third largest peak The second largest peak occurred in fraction 64 In order to compare the protein profile with the activity, the fractions which contained detectable protein are listed in Table 3 5 6 2 3 and the activity of the associated fractions are also shown

Table 3.5.6.2.3 Comparison of protein peaks and biological activity

PEAK	Fr No	A_{280}	Mol wt	ASSAY 1	ASSAY 2	SFMs	SFMc
1	36	0 115	$2x10^6$	92 8 ± 4 2	85 2 ± 4 0	95 5 ± 3 1	111 ± 6 7
2	47	0 047	$66x10^3$	63 1 ± 5 7	48 7 ± 5 2	121 ± 14	108 ± 3 5
3	52	0 024	$31\ 6x10^3$	86 3 ± 8 1	103 ± 8 6	84 4 ± 7 1	106 ± 9 3
4	59	0 022	$12\ 4x10^3$	152 ± 11	150 ± 7 9	107 ± 12	106 ± 7 9
5	64	0 059	$6\ 3x10^3$	------	------	99 4 ± 12	99 4 ± 2 0
6	71	0 051	$2\ 4x10^3$	146 ± 11	308 ± 27	90 5 ± 2 4	90 8 ± 4 0
7	74	0 031	$1\ 78x10^3$	114 ± 3 2	120 ± 7 5	95 3 ± 8 1	83 9 ± 5 5
8	77	0 034	$1\ 26x10^3$	107 ± 7 5	122 ± 16	87 2 ± 5 2	96 7 ± 9 7

Results for activity are expressed as the average percentage growth relative to control (1% DHS for Assay 1 and 2 and SFMs and SFMc only for the two serum-free) ± standard deviation (n=5 for Assays 1 and 2, n=4 for SFMs and SFMc) Abbreviations A_{280} = absorbance at 280nm Fr No = fraction number Mol wt = molecular weight

The results show that where most of the protein eluted off the column (fraction 36), no significant activity either stimulatory or inhibitory was seen for any of the assay systems In fraction 47, where albumin was expected to elute off, inhibition seen in the two low serum assays, and only slight stimulation occurred in the serum-free assays The fact that no activity was obtained for fraction 64 in the low serum assay as this fraction had the second highest absorbance Fraction 71 contained the third largest amount of protein and coincided with activity in the low serum assay, but no activity was seen in the serum-free assays

As a result of one gel filtration experiment some activity was seen in two assays carried out in low serum medium Some stimulation was seen in the serum-free assays but this did not coincide with the stimulation in the low serum assays The simpler of the SFM showed better stimulation than the more complex SFM even though the 0 5M NaCl fraction had shown inhibition in SFM in section 3 5 4 18

It was necessary to repeat the procedure to see if the same trend was obtained A fresh column was set up and re-calibrated The void volume was found to be fraction 38

3.5.6.3 Repeat of Gel Filtration

The results of a second gel filtration with the 0 5M NaCl fraction are shown in Figure 3 5 6 3 1 and 3 5 6 3 2 and Table 3 5 6 3 The activity for the control 0 5M NaCl fraction was low, with 14% and 37 5% stimulation above the control (1% DHS) for the two low serum-supplemented assays Activity was seen in fractions 73 - 79 with maximum stimulation of about 2-fold stimulation over the control occurring in fraction 76 (a molecular weight of less than 2,000 by extrapolation from Figure 3 5 6 2 4) Inhibition was seen in fractions 34 - 40, with no inhibition appearing at fractions 47 to 49 where the albumin should be eluting off The protein profile showed that most of the protein eluted off the column in fraction 47 No activity was seen in fraction 47, but 46 showed 60% stimulation above the control (1% DHS) in the low serum assays

As the simpler of the two SFM showed the best results in 3 5 6 2, the SFMs was used to assay fractions for this filtration (Figure 3 5 6 3 2) Unlike the first filtration, little or no stimulation was seen in the control, only 12 - 31% stimulation above control (SFMs) Together with the results obtained in section 3 5 4 18, it would appear that the stimulation seen in the first experiment may have been due to an artifact

In the first gel filtration, no activity was seen in fraction 46 Both filtrations showed no stimulation occurring in fraction 47 where the albumin should have eluted off the column Activity was seen in fractions 58 - 60 (mol wt 18,200 - 13,800) for the first but not the second gel filtration using low serum-supplemented assays The best activity seen in fractions from the second gel filtration occurred in fractions 76 - 79 (mol wt less than 2,000)

By extrapolating from the calibration curve, the molecular weight of any of the fractions higher than 71 had molecular weights of about 2000 or less However, when the 0 5M NaCl fraction was diafiltered in ATCC during initial preparation, diafiltration occurred using a YM3 (molecular cut off of 3,000) So anything smaller than 3,000 which was not tightly bound to albumin would have been lost Unless gel filtration caused some tightly bound factor to become unbound during passage through the column, the stimulation here would appear to be due to artifacts as there was little activity in the 0 5M NaCl control Both gel filtrations showed some stimulation associated with a factor of less than 2,000 molecular weight

Table 3.5.6.3 Biological activity of fractions from Gel filtration 1 of 0 5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	Assay 1 (SF)	Assay 2 (SF)	A_{280nm}
Control	100 0 ± 7 82	100 0 ± 8 72	100 0 ± 2 70	100 0 ± 6 42	0 000
31	106 2 ± 11 0	111 7 ± 11 5	102 7 ± 3 90	102 9 ± 4 85	0 015
32	96 59 ± 6 56	110 9 ± 6 74	-----	------	0 004
33	88 64 ± 12 1	115 2 ± 10 8	101 6 ± 3 96	101 6 ± 3 47	0 014
34	69 09 ± 5 70	82 70 ± 7 21	------	------	0 033
35	66 36 ± 1 02	57 30 ± 5 01	103 5 ± 2 03	100 6 ± 4 95	0 064
36	66 82 ± 6 93	50 46 ± 10 5	------	------	0 091
37	60 91 ± 1 90	48 85 ± 2 65	102 3 ± 2 73	101 3 ± 4 83	0 074
38	62 30 ± 4 13	50 11 ± 1 49	------	------	0 084
39	60 45 ± 3 45	54 50 ± 2 66	103 5 ± 4 70	99 67 ± 4 90	0 078
40	61 14 ± 3 60	59 90 ± 1 99	105 1 ± 4 52	99 01 ± 10 1	0 051
42	103 3 ± 5 71	106 4 ± 7 14	102 3 ± 4 64	102 3 ± 2 83	0 035
43	105 3 ± 5 17	113 5 ± 5 36	103 5 ± 3 25	104 6 ± 7 44	0 049
44	102 3 ± 7 14	113 2 ± 3 57	96 85 ± 3 01	102 3 ± 5 56	0 041
45	98 68 ± 3 01	109 6 ± 3 57	102 3 ± 4 82	95 30 ± 6 23	0 036
46	142 9 ± 11 1	150 0 ± 9 82	98 09 ± 4 56	94 70 ± 7 40	0 043
47	90 91 ± 8 11	96 40 ± 12 5	99 20 ± 6 36	97 83 ± 4 63	0 021
48	92 98 ± 6 85	97 50 ± 1 07	95 80 ± 2 60	95 36 ± 3 92	0 004
49	98 56 ± 4 97	101 7 ± 8 93	97 30 ± 2 61	94 70 ± 5 94	0 000
50	100 9 ± 5 71	116 0 ± 8 93	95 04 ± 0 76	95 97 ± 5 00	-0 004
51	73 93 ± 8 04	90 50 ± 4 95	95 40 ± 3 81	98 76 ± 6 58	-0 009
52	92 13 ± 12 7	106 9 ± 9 30	95 80 ± 3 15	97 53 ± 6 09	-0 003
53	102 8 ± 6 63	103 8 ± 3 93	101 4 ± 6 28	95 37 ± 6 26	-0 010
54	97 31 ± 2 19	93 02 ± 7 48	103 8 ± 4 31	105 7 ± 4 41	-0 015
55	106 9 ± 15 1	100 0 ± 12 8	101 1 ± 3 91	102 8 ± 8 57	-0 002
56	96 21 ± 12 1	101 0 ± 4 82	100 0 ± 5 58	98 93 ± 5 87	-0 017
57	105 5 ± 4 38	86 20 ± 4 19	97 60 ± 5 97	104 9 ± 6 51	-0 013
58	104 3 ± 11 3	101 2 ± 8 63	96 39 ± 1 81	102 8 ± 3 74	0 037
59	88 72 ± 7 75	100 3 ± 3 84	100 4 ± 1 86	111 0 ± 5 33	-0 003
60	90 24 ± 9 81	98 84 ± 9 36	99 64 ± 2 36	108 9 ± 3 77	-0 011
0 5Mc	114 2 ± 5 30	137 5 ± 10 7	104 6 ± 5 36	100 0 ± 10 6	

Results are expressed as the average percentage growth relative to control (1% DHS for assays 1 and 2(LS), serum-free medium for Assays 1 and 2(SF)) ± standard deviation (n = 5 for low serum assays (DE as end point) and n = 4 for serum-free assays (AP as end point)) The absorbance was read at 280nm against DME phenol red-free medium Abbreviations LS = low serum, SF = serum-free, 0 5Mc = control 0 5M NaCl fraction

Table 3.5.6.3continued Biological activity of fractions from Gel filtration 1 of 0 5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	Assay 1 (SF)	Assay 2 (SF)	A_{280nm}
Control	100 0 ± 6 29	100 0 ± 6 90	100 0 ± 2 90	100 0 ± 5 68	0 000
61	100 0 ± 9 18	107 9 ± 5 51	103 3 ± 5 59	116 7 ± 10 5	0 002
62	108 7 ± 7 82	104 7 ± 11 1	97 45 ± 2 06	98 95 ± 2 41	0 007
63	103 3 ± 14 9	105 3 ± 5 7	97 45 ± 2 37	97 69 ± 4 66	0 003
64	87 43 ± 11 1	94 60 ± 14 2	98 91 ± 2 65	96 74 ± 4 29	0 002
65	102 7 ± 7 12	105 0 ± 9 43	100 0 ± 1 39	96 74 ± 4 53	-0 004
66	108 7 ± 7 82	113 7 ± 9 32	97 82 ± 5 82	102 1 ± 4 71	-0 003
67	112 0 ± 10 9	106 8 ± 11 6	97 09 ± 1 39	97 69 ± 3 64	0 010
68	114 2 ± 13 6	98 20 ± 19 4	100 0 ± 3 22	103 0 ± 6 21	0 008
69	103 8 ± 9 85	87 00 ± 19 0	95 27 ± 1 86	98 32 ± 5 14	-0 006
70	100 5 ± 9 34	82 40 ± 7 18	93 82 ± 1 45	101 5 ± 3 17	-0 004
71	100 0 ± 6 34	127 0 ± 13 2	101 8 ± 4 49	101 7 ± 8 74	-0 006
72	105 6 ± 8 55	138 0 ± 11 0	100 4 ± 1 90	99 64 ± 1 64	-0 005
73	100 9 ± 12 2	142 4 ± 10 9	103 7 ± 3 68	102 8 ± 1 79	-0 011
74	135 8 ± 16 3	156 2 ± 5 38	104 5 ± 6 99	103 2 ± 5 26	-0 010
75	209 8 ± 36 2	204 9 ± 11 7	100 4 ± 4 30	101 1 ± 8 14	-0 006
76	229 7 ± 38 7	201 2 ± 10 5	100 7 ± 4 43	105 3 ± 4 03	-0 005
77	177 8 ± 18 4	210 8 ± 11 8	101 5 ± 2 88	109 9 ± 6 29	-0 015
78	147 3 ± 9 94	186 6 ± 14 1	96 20 ± 3 01	108 2 ± 6 68	-0 010
79	116 5 ± 14 8	156 8 ± 10 2	97 70 ± 6 53	115 3 ± 9 08	-0 010
80	86 90 ± 7 84	144 8 ± 8 91	99 63 ± 3 47	99 35 ± 4 80	0 017
81	125 0 ± 4 46	113 1 ± 7 38	98 90 ± 1 40	102 9 ± 6 65	-0 021
82	142 8 ± 11 8	117 4 ± 6 37	98 50 ± 3 17	100 9 ± 5 64	-0 019
83	147 3 ± 11 8	115 1 ± 9 77	98 20 ± 5 15	96 10 ± 4 50	-0 008
84	146 2 ± 7 62	123 3 ± 5 13	97 06 ± 1 20	103 2 ± 3 44	-0 038
85	120 5 ± 14 1	96 12 ± 1 60	------	------	-0 046
86	97 30 ± 5 82	88 95 ± 6 42	97 06 ± 2 40	99 00 ± 5 23	-0 073
87	121 4 ± 23 5	83 20 ± 8 16	------	------	-0 071
88	129 5 ± 1 63	88 95 ± 3 87	96 16 ± 5 00	103 6 ± 2 67	-0 055
89	111 6 ± 3 64	88 95 ± 3 87	------	------	-0 050
90	115 0 ± 6 98	106 6 ± 7 53	98 16 ± 3 00	100 9 ± 3 88	-0 044

Results are expressed as the average percentage growth relative to control (1% DHS for assays 1 and 2 (LS), serum-free for Assays 1 and 2 (SF)) ± standard deviation (n = 5 for low serum assays (DE as end point) and n = 4 for serum-free assays (AP as end point)) The absorbance was read at 280nm against DME phenol red-free medium Abbreviations LS = low serum, SF = serum-free

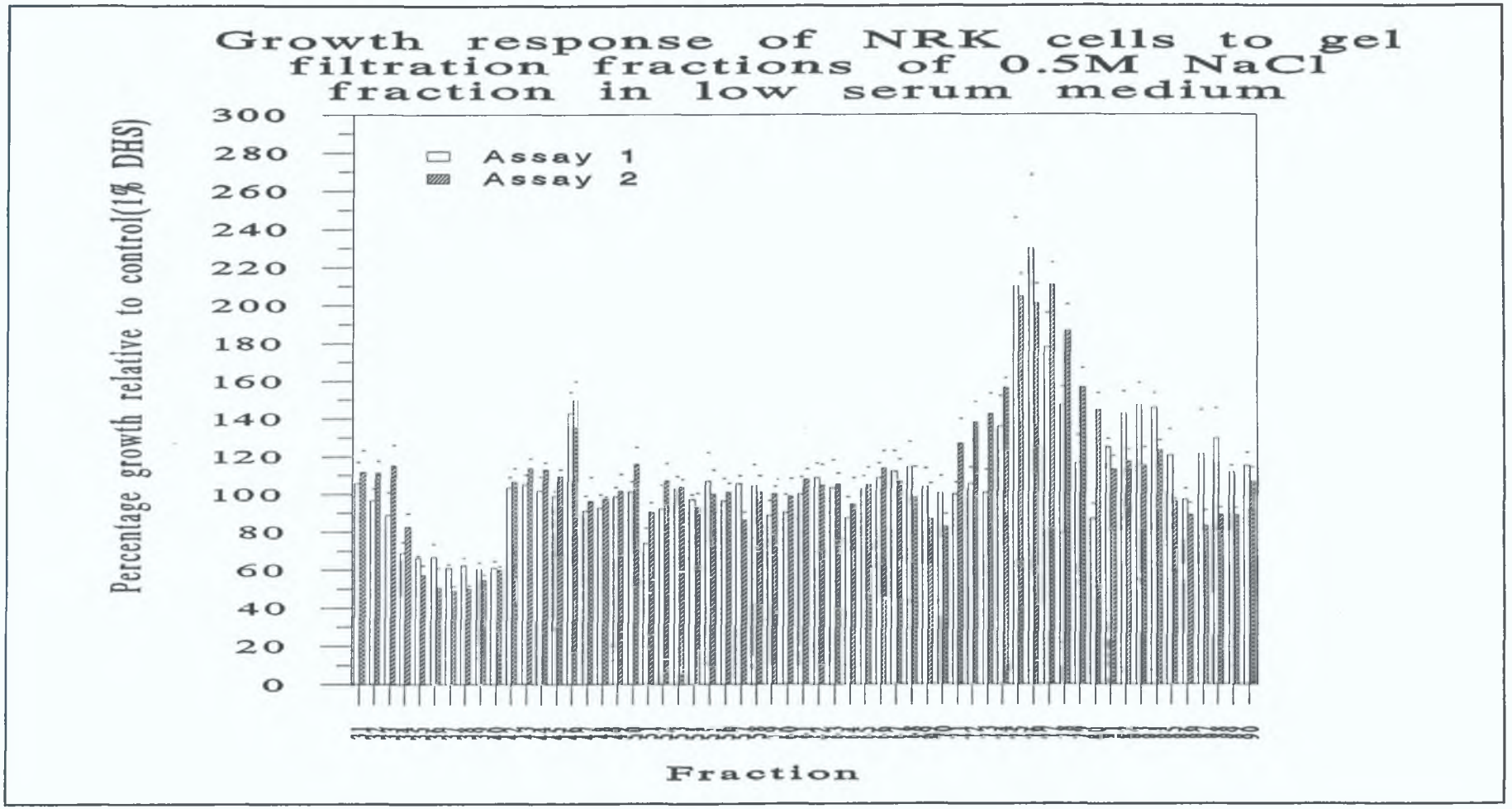


Figure 3.5.6.3.1 Growth response of NRK cells to gel filtration fractions of the 0.5M NaCl fraction in low serum medium. Results are expressed as the average percentage growth relative to control (1% DHS) $\pm$ standard deviation (n=5). Dye elution was used as the end point of two separate assays.

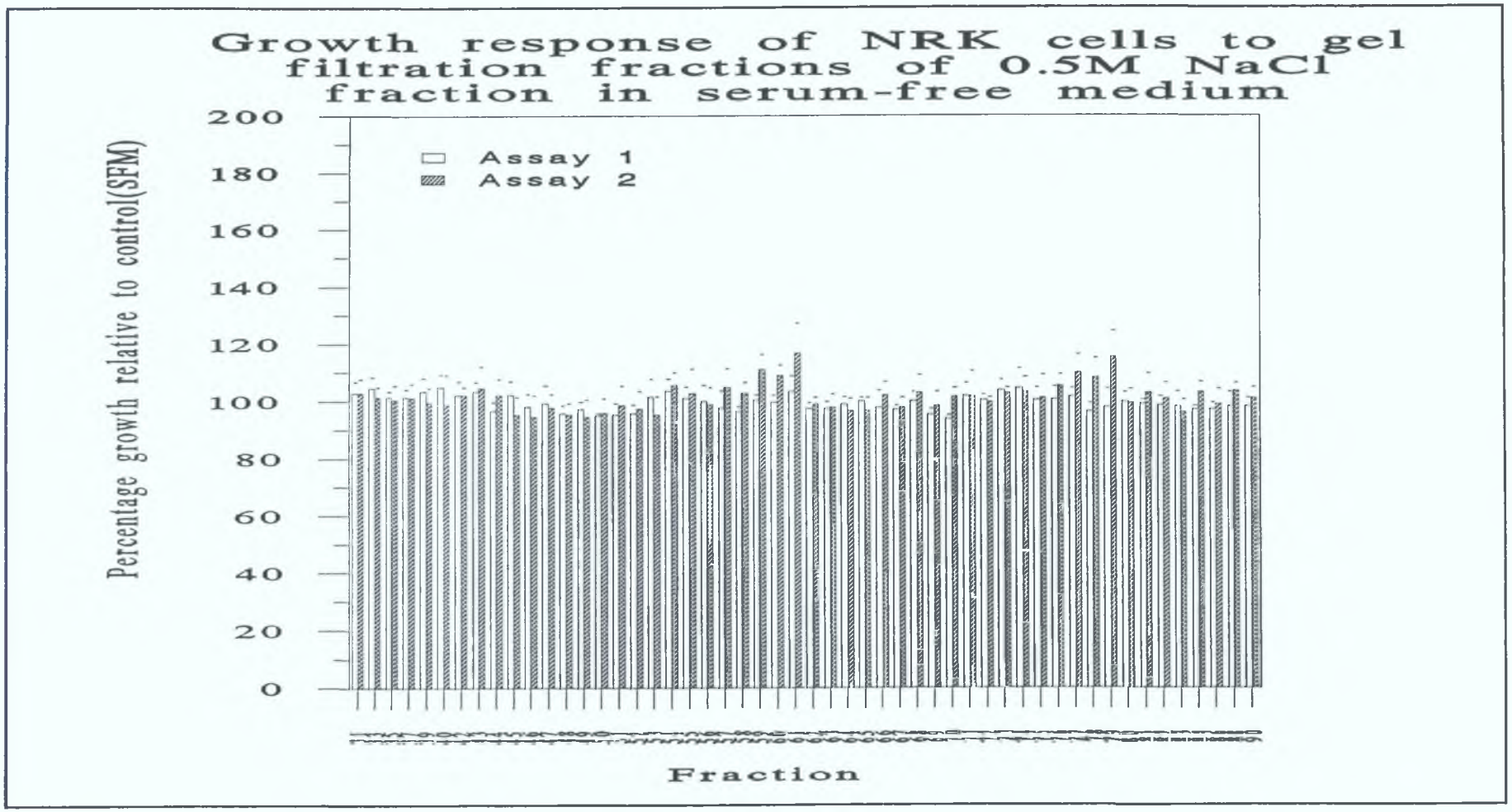


Figure 3.5.6.3.2 Growth response of NRK cells to gel filtration fractions of the 0.5M NaCl fraction in serum-free medium. Results are expressed as the average percentage growth relative to control (SFMs) $\pm$ standard deviation (n=4). Acid phosphatase was used as the end point for separate assays.

3.5.6.4 Gel filtration of BSA

With the low activity obtained with the 0 5M NaCl control and no consistent activity being observed after gel filtration except less than 2,000 molecular weight, it was decided to subject BSA to gel filtration to see where (if any) activity was eluting from the column

In low serum-supplemented assays, slight stimulation was seen but no significant peak of activity was observed (Figure 3 5 6 4 1) In fraction 47, BSA was growth inhibitory relative to the control (1% DHS) by about 10 - 20%

Good stimulation was seen in assay 2 where the control albumin showed a 2 2-fold stimulation over the control (1% DHS) In assay 1, the control BSA was only 58% stimulatory above the control There appeared to be less stimulation overall in assay 1 Although there was stimulation in several fractions (62-63, 85-87 and 88-100), none individually were as good as the control BSA

When the fractions were tested in the serum-free medium (SFMs), variable responses to the fractions were seen depending on the assay (Figure 3 5 6 4 2) No stimulation was seen in fraction 47 or in the BSA control (No activity for BSA at 0 1mg/ml was also seen in section 3 5 4 18)

According to the protein profile of the BSA, one major protein peak occurred in fraction 47 (Figure 3 5 6 4 2) A small spike occurred in fraction 42, probably due to aggregation of albumin No stimulation and about 20% inhibition was seen in fractions 42 and 47 for low serum assays respectively and no effect was observed in serum-free assays

Table 3.5.6.4 Biological activity of fractions from Gel filtration 1 of 0.5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	Assay 1 (SF)	Assay 2 (SF)	A_{280nm}
Control	100.0 ± 6.14	100.0 ± 8.82	100.0 ± 14.7	100.0 ± 6.42	0.000
35	63.38 ± 2.47	84.31 ± 5.80	157.9 ± 21.0	88.98 ± 4.72	0.014
36	71.51 ± 6.12	90.65 ± 8.32	125.7 ± 10.1	83.99 ± 2.10	0.004
37	55.72 ± 1.88	73.91 ± 3.69	122.8 ± 30.1	94.48 ± 12.2	0.006
38	76.77 ± 2.34	94.12 ± 4.04	278.5 ± 48.3	85.56 ± 4.24	0.005
39	84.89 ± 8.28	109.5 ± 2.22	155.7 ± 8.07	92.91 ± 4.89	-0.055
40	102.9 ± 10.1	111.9 ± 8.87	161.4 ± 15.3	95.01 ± 5.78	0.104
41	98.97 ± 8.79	114.8 ± 2.29	151.3 ± 9.99	93.96 ± 5.03	0.344
42	97.25 ± 5.41	115.9 ± 12.0	159.5 ± 10.1	95.80 ± 5.45	0.605
43	92.79 ± 5.64	109.3 ± 6.89	173.0 ± 12.9	101.0 ± 4.23	0.496
44	84.83 ± 3.32	105.6 ± 4.39	95.51 ± 8.68	78.78 ± 3.13	0.289
45	97.25 ± 4.33	105.4 ± 11.5	95.74 ± 14.3	80.12 ± 3.95	0.646
46	87.47 ± 5.03	118.7 ± 9.84	95.74 ± 4.65	81.90 ± 3.21	1.220
47	82.48 ± 5.22	79.78 ± 3.86	100.0 ± 8.19	81.90 ± 0.73	1.531
48	74.72 ± 11.3	98.26 ± 31.8	95.96 ± 8.82	83.01 ± 7.49	0.341
49	85.34 ± 16.8	122.3 ± 14.2	102.7 ± 6.32	83.90 ± 1.97	1.125
50	83.29 ± 14.7	123.3 ± 14.5	111.3 ± 10.9	90.87 ± 3.08	0.808
51	94.09 ± 12.8	126.2 ± 27.6	104.5 ± 5.50	83.45 ± 4.50	0.482
52	93.99 ± 18.4	103.6 ± 20.8	104.0 ± 2.64	87.68 ± 3.74	0.170
53	85.00 ± 13.9	87.12 ± 7.01	109.3 ± 8.34	89.98 ± 7.45	0.075
54	115.2 ± 7.07	108.8 ± 5.48	115.7 ± 11.3	87.75 ± 4.51	-0.011
55	114.7 ± 7.53	107.2 ± 3.54	120.4 ± 11.9	87.97 ± 4.07	0.013
56	115.3 ± 4.53	110.8 ± 8.06	103.6 ± 5.12	90.87 ± 6.38	0.016
57	103.9 ± 5.70	107.4 ± 8.86	101.6 ± 6.75	89.53 ± 4.68	0.006
58	107.2 ± 14.0	107.4 ± 1.88	101.6 ± 7.65	86.19 ± 4.07	-0.011
59	111.9 ± 3.04	119.6 ± 15.8	105.4 ± 5.4	87.08 ± 4.56	-0.054
60	111.9 ± 7.35	114.9 ± 3.71	123.2 ± 17.3	87.28 ± 3.81	-0.052
61	111.9 ± 3.06	113.7 ± 11.6	105.5 ± 18.9	92.80 ± 8.56	-0.026
62	113.8 ± 6.29	123.7 ± 8.25	103.3 ± 10.3	92.80 ± 6.98	-0.037
63	113.8 ± 5.53	124.5 ± 14.8	101.4 ± 9.62	93.52 ± 8.95	-0.023
64	106.0 ± 7.49	123.0 ± 12.4	111.4 ± 2.92	98.32 ± 1.66	-0.015

Results are expressed as the average percentage growth relative to control (1% DHS for assays 1 and 2, SFMs and SFMc only for serum-free assays) ± standard deviation (n = 5 for low serum assays (DE as end point) and n = 4 for serum-free assays (AP as end point)). The absorbance was read at 280nm against DME phenol red-free medium. Abbreviations: LS = low serum; SF = serum-free.

Table 3.5.6.4continued Biological activity of fractions from Gel filtration 1 of 0 5M NaCl

Fraction	Assay 1 (LS)	Assay 2 (LS)	Assay 1 (SF)	Assay 2 (SF)	A_{280nm}
Control	100 0 ± 9 94	100 0 ± 12 4	100 0 ± 2 92	100 0 ± 9 04	0 000
65	112 0 ± 8 92	138 8 ± 9 79	94 44 ± 12 6	74 82 ± 4 07	-0 048
66	119 3 ± 8 24	142 5 ± 23 1	96 11 ± 9 14	79 86 ± 2 52	-0 027
67	112 6 ± 6 70	132 5 ± 6 41	110 8 ± 16 5	83 93 ± 1 84	-0 020
68	120 3 ± 10 6	132 2 ± 10 8	94 72 ± 8 72	79 86 ± 2 52	-0 019
69	98 88 ± 1 14	125 8 ± 13 4	101 4 ± 4 29	77 94 ± 6 13	-0 032
70	96 63 ± 10 6	11 1 ± 12 0	91 10 ± 1 20	88 70 ± 4 33	-0 041
71	101 7 ± 6 06	118 5 ± 8 20	93 92 ± 11 7	89 90 ± 5 94	-0 017
72	88 49 ± 13 1	120 3 ± 7 41	101 2 ± 13 8	85 28 ± 7 22	-0 015
73	85 55 ± 16 4	120 7 ± 9 19	100 0 ± 10 5	87 90 ± 9 00	-0 029
74	100 0 ± 8 44	111 3 ± 9 63	91 82 ± 5 93	82 46 ± 6 46	-0 008
75	100 4 ± 12 0	127 8 ± 10 7	106 3 ± 14 3	84 30 ± 6 76	-0 016
76	102 4 ± 4 94	123 9 ± 9 77	113 5 ± 14 2	83 30 ± 4 02	-0 026
77	86 47 ± 5 76	111 3 ± 5 15	120 6 ± 13 2	90 50 ± 8 99	-0 023
78	110 7 ± 5 72	102 6 ± 7 92	127 6 ± 3 45	90 90 ± 3 63	-0 053
79	108 4 ± 7 23	108 2 ± 12 9	95 79 ± 6 39	92 39 ± 13 2	-0 024
80	101 7 ± 10 1	119 6 ± 12 1	96 00 ± 3 92	94 62 ± 4 48	-0 034
82	98 42 ± 6 85	113 2 ± 10 4	92 90 ± 2 60	86 96 ± 8 74	-0 020
83	104 3 ± 9 83	112 8 ± 10 7	99 78 ± 7 13	94 56 ± 13 2	-0 023
84	78 85 ± 6 96	95 12 ± 7 89	90 93 ± 8 59	101 6 ± 5 06	-0 034
85	107 3 ± 8 68	137 8 ± 12 2	88 94 ± 8 83	104 0 ± 6 72	-0 015
86	118 4 ± 6 09	133 9 ± 14 4	93 58 ± 7 41	103 8 ± 5 14	-0 022
87	116 1 ± 8 49	138 8 ± 21 3	98 67 ± 5 28	100 8 ± 7 28	-0 003
88	111 5 ± 9 71	107 8 ± 11 3	101 3 ± 4 12	102 7 ± 7 86	-0 015
89	115 9 ± 11 5	153 4 ± 11 6	102 8 ± 3 54	101 9 ± 1 05	-0 020
90	113 3 ± 11 1	147 8 ± 6 21	98 00 ± 9 09	103 0 ± 10 1	-0 032
92	115 3 ± 14 4	142 4 ± 6 99	100 3 ± 2 82	97 81 ± 10 2	-0 003
96	113 6 ± 8 69	152 0 ± 16 7	140 3 ± 28 3	94 53 ± 8 01	-0 040
98	114 8 ± 6 23	151 2 ± 15 7	99 78 ± 5 18	112 0 ± 3 15	-0 011
100	99 64 ± 12 2	141 5 ± 21 7	95 20 ± 7 86	112 3 ± 5 81	-0 007
BSAc	158 0 ± 14 1	224 5 ± 13 7	102 6 ± 2 95	96 50 ± 9 89	-0 007

Results are expressed as the average percentage growth relative to control (1% DHS for assays 1 and 2, SFMs and SFMc only for serum-free assays) ± standard deviation (n = 5 for low serum assays (DE as end point) and n = 4 for serum-free assays (AP as end point)) The absorbance was read at 280nm against DME phenol red-free medium Abbreviations LS = low serum, SF = serum-free

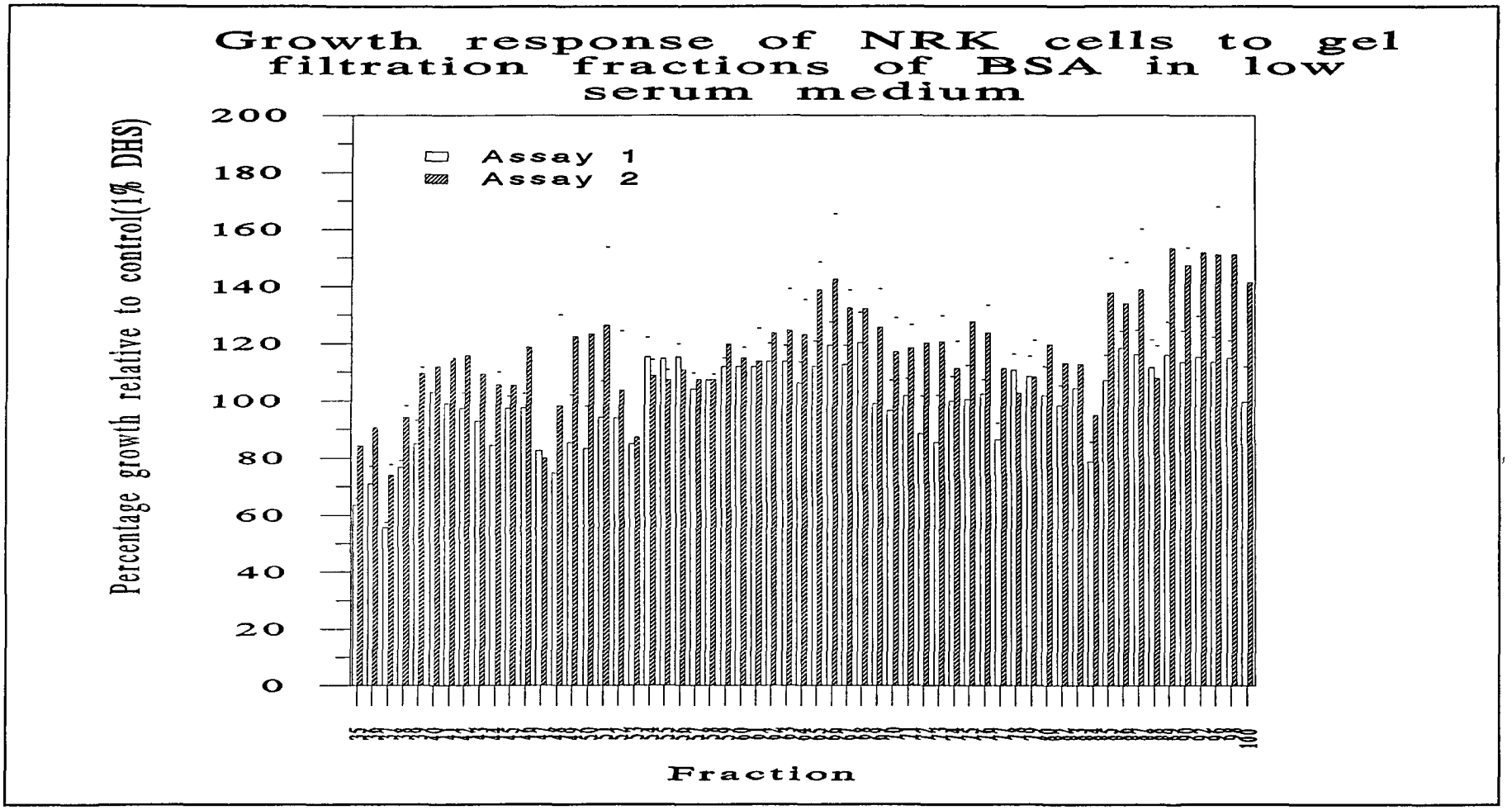


Figure 3.5.6.4 1 Growth response of NRK cells to gel filtration fractions of BSA in low serum medium Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=5) Dye elution was used as the end point for two separate assays

These experiments showed that for the 0 5M NaCl fraction, two separate gel filtration runs showed stimulating activity at low molecular weights of less than 2,000 A protein peak corresponded with the activity in one experiment but not in the other

The low activity may have been due to the activity being spread out over several fractions as a result may have lowered the individual activity of a fraction The active factor may also have required the presence of the BSA molecule to elicit its activity However, the control for the 0 5M NaCl fraction was low

When the untreated BSA was run on the column, there was a spread of activity over the fractions This activity was low and did not correspond to one main peak suggesting that a variety of factors may be required to impart the activity to BSA There was no activity at the position at which the albumin was eluted from the column In addition, no peak activity was seen at low molecular weights of less than 2,000 to correspond to the peaks found with the 0 5M NaCl fraction

A 9,000 molecular weight protein such as erythrotropin-like factor would be eluted from the column around fraction 61 For the 0 5M NaCl fraction, there was little or no activity seen in fraction 61, with some stimulation in fractions 62 and 63 in one experiment and no activity in the second experiment No significant activity was seen in these fractions with the BSA control These results indicate that if erythrotropin-like factor was present on the albumin, it did not account for the activity associated with the BSA or the 0 5M NaCl fraction, unless its activity required the presence of the BSA molecule

3.5.6.5 DIAFILTRATION OF BSA

In order to investigate the possibility that recombination of factors/components of different molecular weight were necessary to restore activity, BSA was sequentially diafiltered through a series of membranes with decreasing molecular weight cut offs (100,000, 30,000, 10,000 and 3,000) The filtrates (F_{100}, F_{30}, F_{10} and F_3) and retentates (R_{100}, $R_{30\text{-}100}$, $R_{10\text{-}30}$ and $R_{3\text{-}10}$) were tested alone and in combination for their growth stimulatory activity on NRK cells in low serum conditions In two separate diafiltration experiments, a 20mg/ml BSA solution was diafiltered A second solution with just ATCC was diafiltrated as a control for factors in the ATCC which may have been isolated in any of the retentates

The results for two separate diafiltrations (two assays per diafiltration) are shown in Tables 3 5 6 5 1a and b In the first diafiltration, variable activity was retained in the R_{100} (only 10% in assay 1 and 58 3% in assay 2 of the activity seen with the 1% DHS control) 83-91% stimulation above the control (1% DHS) was seen in the filtrate (F_{100}) This corresponded to 66% of the activity obtained with the BSA control Recombination of the retentate and filtrate was slightly more stimulatory than the filtrate alone, resulting in 76 - 82% of the activity obtained by the BSA control In the second diafiltration, 53 - 67 6% of the activity obtained by the control BSA was retained in the R_{100} The filtrate showed the same stimulation as the BSA control (100 - 121% of the activity found in the control BSA) The combination of the filtrate and retentate was again as stimulatory as the BSA control (116 9 - 81%)

For first diafiltration, the $R_{30\text{-}100}$ showed 47 - 55% of the growth achieved by the BSA control In the first assay, only 21% activity was retained in the $F_{30}+R_{30\text{-}100}$ In the second diafiltration, activity was seen in the retentate but little or no stimulation in the filtrate The amount of activity in the $R_{30\text{-}100}$ was variable between the two assays, 35 - 73% of that obtained with the BSA control Recombination of the $R_{30\text{-}100}$ and F_{30} increased the activity to 48 6 - 82 7% of that obtained with the BSA control For the $R_{10\text{-}30}$ and F_{10} little or no stimulation was seen in either diafiltration (no F_{10} was kept for assaying in diafiltration 2) Some slight inhibition was seen in the $R_{3\text{-}10}$ and F_3 in the first diafiltration but not in the second

The diafiltered ATCC controls from the first diafiltration were assayed to ensure that components from the medium were not responsible for the observed results Little or no stimulation was seen when the standard deviations were taken into account (Table 3 5 6 5 2) Slight inhibition by $R_{3\text{-}10}$ was seen in one of the two assays

Table 3.5.6.5.1a Biological activity of retentates and filtrates of BSA (Diafiltration 1)

VARIABLE	ASSAY 1	ASSAY 2	VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 5 39	100 0 ± 10 6	1% DHS	100 0 ± 5 95	100 0 ± 10 6
+ BSA	237 6 ± 32 0	223 9 ± 14 2	+ BSA	209 1 ± 13 9	223 9 ± 14 2
+ R_{100}	112 9 ± 4 99	158 3 ± 14 0	+ $R_{10\ 30}$	118 8 ± 2 75	96 36 ± 8 50
+ F_{100}	190 6 ± 21 5	182 6 ± 20 8	+ F_{10}	100 0 ± 18 4	78 54 ± 9 50
+R_{100}+F_{100}	204 7 ± 40 0	158 7 ± 17 0	+ R+F_{10}	81 90 ± 10 3	73 68 ± 8 50
+ $R_{30\ 100}$	174 1 ± 20 1	158 7 ± 11 3	+ $R_{3\ 10}$	70 13 ± 5 50	100 6 ± 8 06
+ F_{30}	101 2 ± 4 03	70 99 ± 10 9	+ F_3	89 61 ± 10 3	101 6 ± 6 52
+ $R_{30\ 100}$+F_{30}	129 4 ± 12 4	105 5 ± 14 7	+ R+F_3	75 97 ± 2 75	97 70 ± 4 06

Table 3.5.6.5.1b Biological activity of retentates and filtrates of BSA (Diafiltration 2)

VARIABLE	ASSAY 1	ASSAY 2	VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 10 6	100 0 ± 7 59	1% DHS	100 0 ± 10 6	100 0 ± 8 00
+ BSA	233 8 ± 16 7	233 0 ± 22 2	+ BSA	233 8 ± 16 7	233 0 ± 22 2
+ R_{100}	190 4 ± 14 5	170 6 ± 14 1	+ $R_{10\ 30}$	154 5 ± 13 9	113 7 ± 5 88
+ F_{100}	262 1 ± 27 7	233 0 ± 12 0	1% DHS	100 0 ± 7 86	100 0 ± 9 20
+R+F_{100}	256 4 ± 25 8	207 8 ± 21 1	+ BSA	246 0 ± 17 9	223 0 ± 8 00
+ $R_{30\ 100}$	198 3 ± 10 0	147 0 ± 13 8	+ $R_{3\ 10}$	102 6 ± 7 86	97 67 ± 9 30
+ F_{30}	137 6 ± 19 6	109 8 ± 9 60	+ F_3	100 0 ± 7 86	83 72 ± 6 97
+ R+F_{30}	210 7 ± 25 3	164 7 ± 9 19	+ R+F_3	92 30 ± 10 2	86 00 ± 9 30

Table 3.5.6 5.2 Biological activity of retentates and filtrates of ATCC

VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 28 0	100 0 ± 19 3
+ BSA	294 1 ± 20 4	244 0 ± 27 0
+ R_{100}	104 4 ± 15 5	102 0 ± 25 4
+ F_{100}	91 20 ± 6 74	126 0 ± 27 5
+ $R_{30\ 100}$	91 20 ± 15 5	102 0 ± 20 8
+ F_{30}	99 99 ± 9 18	122 2 ± 15 1
+ $R_{10\ 30}$	101 4 ± 7 64	108 1 ± 1 06
+ F_{10}	94 1 ± 14 2	63 00 ± 0 42
+ $R_{3\ 10}$	74 65 ± 4 88	93 30 ± 8 31
+ F_3	108 4 ± 12 9	93 30 ± 15 4

The results for the combinations of the retentates are shown in Table 3 5 6 5 3 The retentates from the ATCC diafiltrations were also recombined These were referred to as for example $R_{3\text{-}10}+R_{100}$ATCC, while the BSA solutions were referred to $R_{3\text{-}10}+R_{100}$BSA

While the control $R_{10\text{-}30}+R_{3\ 10}$ATCC showed no stimulation, $R_{10\text{-}30}+R_{3\text{-}10}$BSA showed between 28 1 and 42 2% stimulation above the control (1% DHS) in both diafiltrations (except assay 2 in Diafiltration 1, 2 2%) The amount of stimulation was low varying between 20 6 - 25 4% of the activity of the BSA control (Again very low activity was seen in Assay 2 from Diafiltration 1)

The combination $R_{30\text{-}100}+R_{3\text{-}10}$BSA was not as stimulatory as the control BSA The ATCC control was inhibitory in the first diafiltration but had no effect in the second diafiltration

For the combination of $R_{100}+R_{3\text{-}10}$BSA, improved growth in the first diafiltration but not in the second was seen The addition of $R_{3\text{-}10}$ did not improve growth obtained with the R_{100} alone

Table 3.5.6.5.3a Biological activity combinations of retentates of BSA (Diafiltration 1)

VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 5 39	100 0 ± 4 90
+ BSA	304 2 ± 27 7	243 2 ± 15 9
+ $R_{10\ 30}$ + $R_{3\ 10}$BSA	142 2 ± 12 2	102 2 ± 11 3
+ $R_{10\ 30}$ + $R_{3\ 10}$ATCC	97 18 ± 26 4	106 6 ± 11 8
+ R_{100} + $R_{3\ 10}$BSA	181 7 ± 12 7	168 2 ± 13 6
+ R_{100} + $R_{3\ 10}$ATCC	94 37 ± 19 0	96 30 ± 6 79
1% DHS	100 0 ± 7 89	100 0 ± 4 90
+ BSA	168 7 ± 0 15	243 2 ± 15 9
+ $R_{30\ 100}$ + $R_{3\ 10}$BSA	156 2 ± 9 44	175 0 ± 6 82
+ $R_{30\ 100}$ + $R_{3\ 10}$ATCC	86 25 ± 11 2	76 00 ± 2 17

Results are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=3)

Table 3.5.6.5.3b Biological activity of combination of retentates of BSA (Diafiltration 2)

VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 7 86	100 0 ± 9 20
+ BSA	246 0 ± 17 9	223 0 ± 16 3
+ R_{100} + $R_{3\ 10}$BSA	171 7 ± 12 8	146 3 ± 13 9
+ R_{100} + $R_{3\ 10}$ATCC	72 51 ± 9 13	83 39 ± 9 84
+ $R_{30\ 100}$ + $R_{3\ 10}$BSA	176 9 ± 5 10	146 5 ± 11 6
+ $R_{30\ 100}$ + $R_{3\ 10}$ATCC	103 0 ± 8 64	75 98 ± 20 5
1% DHS	100 0 ± 7 86	100 0 ± 9 20
+ BSA	246 0 ± 17 9	233 0 ± 16 3
+ $R_{10\ 30}$ + $R_{3\ 10}$BSA	123 1 ± 10 2	111 6 ± 9 30
+ $R_{10\ 30}$ + $R_{3\ 10}$ATCC	102 3 ± 9 88	102 8 ± 11 1

Results are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=8)

The results showed that without recombination of factors, most of the activity was retained above the $R_{30\text{-}100}$ Below this little or no activity was seen The ATCC controls showed that only diafiltration of the BSA and not the ATCC medium was responsible for the activity seen

Results for the combinations as a percentage of the BSA control are shown in Table 3 5 6 5 4 An additional set of assays were carried out on the retentates and these are also shown in Table 3 5 6 5 4 Most of the activity was seen in the R_{100} and $R_{30\text{-}100}$ Little or no activity was seen below this Recombination of the $R_{3\text{-}10}$ did not improve the growth of fractions

Table 3.5.6.5.4 Biological activity of Combinations relative to BSA

VARIABLE	DIAFILTRATION 1				DIAFILTRATION 2			
	ASSAY 1		ASSAY 2		ASSAY 1		ASSAY 2	
R_{100}	9 40	41 6	47 1	58 0	67 5	59 40	53 1	80 4
$R_{30\ 100}$	53 8	59 9	47 4	37 1	73 5	41 20	35 3	28 1
$R_{10\ 30}$	17 2	--	0 00	--	40 7	--	10 3	--
$R_{3\ 10}$	0 0	0 00	0 50	2 22	1 78	0 00	0 00	2 20
$R_{100}+R_{3\ 10}$BSA	40 0	43 7	28 0	51 3	49 1	50 0	37 6	81 8
$R_{30\ 100}+R_{3\ 10}$BSA	81 8	52 9	30 8	31 4	52 7	57 9	37 8	31 4
$R_{10\ 30}+R_{3\ 10}$BSA	20 6	--	1 54	--	15 8	--	9 43	--
$F_{100}+R_{100}$	76 1	--	81 7	--	116 9	--	81 0	--
F_{100}	65 8	--	66 7	--	121 1	--	100 0	--

Results are expressed as the percentage growth relative to untreated BSA $\pm$ standard deviation (n=3 for Diafiltration 1 and n=8 for Diafiltration 2)

The result from both experiments shows that most of the activity is in fractions which contain albumin protein (either as a monomer or polymer) Measurement of the protein concentration of the R_{100} and $R_{30\text{-}100}$ showed a similar protein concentration in both diafiltrations 53% of the protein applied was retained in the R_{100} with 30% being retained in the $R_{30\text{-}100}$ in the first diafiltration In the second diafiltration, 48% was retained in the R_{100} and 22% in the $R_{30\text{-}100}$

3.5.6.6 Diafiltration of 0.5M NaCl Fraction

Diafiltration was carried out using the 0 5M NaCl fraction as described for BSA in section 3 5 6 5 The results for the retentates only are shown in Tables 3 5 6 6 1 and 2

Table 3.5.6.6.1 Biological activity of retentates of the 0 5M NaCl fraction

VARIABLE	ASSAY 1	ASSAY 2
1%DHS	100 0 ± 7 08	100 0 ± 6 20
+ 0 5M NaCl	205 1 ± 15 0	222 2 ± 18 5
+ R_{100}	101 6 ± 3 76	96 82 ± 8 67
+ $R_{30\ 100}$	99 73 ± 5 64	109 2 ± 10 7
+ $R_{10\ 30}$	95 03 ± 5 29	76 54 ± 8 34
+ $R_{3\ 10}$	94 09 ± 7 53	59 88 ± 11 3
+ R_{100} + $R_{3\ 10}$	105 1 ± 10 0	82 09 ± 18 6
+ $R_{30\ 100}$ + $R_{3\ 10}$	98 39 ± 11 6	83 95 ± 22 0
+ $R_{10\ 30}$ + $R_{3\ 10}$	97 85 ± 7 52	62 96 ± 16 1
1% DHS	100 0 ± 6 60	100 0 ± 7 20
+ 0 5M NaCl	275 8 ± 17 2	169 9 ± 20 9
+ R_{100} + $R_{30\ 100}$	155 2 ± 13 8	112 6 ± 11 5
+ R_{100} + $R_{10\ 30}$	168 9 ± 6 89	115 7 ± 13 0
+ $R_{30\ 100}$ + $R_{10\ 30}$	210 3 ± 13 8	157 4 ± 8 50

Results are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=8)

The results show that no apparent activity was visible in any of the retentates This was unlikely to be due to a loss of the active factor on diafiltration, as diafiltration of the 0 5M NaCl fraction did not result in a loss of activity as seen in section 3 5 4 20 The loss of activity may also have been due to a requirement for some albumin to be present for the active factor to elicit a response Recombination of any of the factors with the $R_{3\text{-}10}$ did not result in a recovery of activity However, recombination of the $R_{30\text{-}100}$ + R_{100} and the R_{100} +$R_{10\text{-}30}$ showed 20 to 30% of the activity observed with the 0 5M NaCl fraction alone (Table 3 5 6 6 2) When the $R_{30\text{-}100}$ and $R_{10\text{-}30}$ were recombined, 60 to 80% of the activity associated with the 0 5M NaCl fraction was restored

Table 3.5 6.6 1 Biological activity of retentates of the 0 5M NaCl fraction

VARIABLE	ASSAY 1	ASSAY 2
+ 0 5M NaCl	100 0	100 0
+ R_{100}	1 5	0 00
+ $R_{30\ 100}$	0 00	7 38
+ $R_{10\ 30}$	0 00	0 00
+ $R_{3\ 10}$	0 00	0 00*
+ R_{100} + $R_{3\ 10}$	4 70	0 00*
+ $R_{30\ 100}$ + $R_{3\ 10}$	0 00	0 00*
+ $R_{10\ 30}$ + $R_{3\ 10}$	0 00	0 00*
+ R_{100} + $R_{10\ 30}$	31 4	18 0
+ R_{100} + $R_{30\ 100}$	39 2	22 5
+ $R_{10\ 30}$ + $R_{30\ 100}$	62 7	82 2

Results are expressed as the percentage growth relative to 0 5M NaCl fraction

The results for the diafiltration of the 0 5M NaCl fraction suggest that the activity of the 0 5M NaCl fraction was not due to a subpopulation of albumin as no activity was seen in the $R_{30\text{-}100}$ Moreover, the activity appeared on recombination of the $R_{30\text{-}100}$ and the $R_{10\text{-}30}$ Activity was also seen in the combination of the R_{100} with either the $R_{10\text{-}30}$ or $R_{30\text{-}100}$ These results show a trend different to that observed with the native albumin on diafiltration

CHAPTER FOUR

DISCUSSION

4.0 DISCUSSION

Serum is a requirement of virtually all types of cells grown in culture, its function being to provide the additional nutrients and protective factors not present in the basal medium Fetal bovine / calf sera are most commonly used to support growth However, problems in the use of serum include cost, batch variability, supply limitations and the potential of biological contamination (viruses and mycoplasma) The undefined nature of serum makes interpretation of results difficult and hampers the elucidation of biochemical pathways On an industrial scale, additional problems exist The variability in serum batches results in variable production runs The high levels of protein present in serum make downstream processing more difficult and increases purification costs Also, regulations governing the production of pharmaceuticals are critical of the excessive use of serum in industrial production

To overcome many of the above problems, serum-free media (SFM) were developed However, as the nutritional requirements varied considerably between cell lines and culture conditions, not to mention the complexity of serum itself, it has not been possible to develop a universal SFM which would support the growth of all cell lines

In order to encourage cell growth, many growth factors and hormones normally present in serum were added Two of the most commonly used supplements in SFM are bovine insulin and human transferrin While growth in SFM has overcome many of the problems mentioned above, the use of animal-derived proteins still present the possibility of biological contamination in addition to a loss of media definition For these reasons and to satisfy industrial regulations, development of protein free / defined media has become important Production of proteins from such a medium would, theoretically, be free from immunoglobulins and other protein contaminants associated with serum-derived components such as albumin and transferrin

The development of protein-free media is usually based on supplementing the basal medium with a wide variety of low molecular weight components to such an extent that cells do not require large molecular weight proteins Most success has been achieved for anchorage-independent cells which are in general more tolerant and have less demanding nutritional requirements (Agy *et al* , 1981, Darfler, 1990, Shinmoto *et al* , 1988) than anchorage-dependent cells Important growth stimulatory factors may not be required for some cell lines which exhibit an autocrine production system (Rikimaru *et al* 1990) Where a dependence on

exogenously added growth factors or hormones is required, recombinant or synthetic analogues (*e g* α-cyclodextrin and cholesterol for albumin, ferric-gluconate for transferrin) have been employed Recombinant growth factors are sometimes used it is claimed that they are purer than serum-derived proteins

It was the aim of this project to investigate the serum-derived proteins and in serum-free medium so as to replace them with non animal-derived components which may take the form of recombinant proteins / synthetic analogues or simple chemical compounds (*e g* recombinant insulin or IGF-I and ferric complexes in the case of insulin and transferrin respectively)

In order to do this, it was necessary to be able to screen a wide range of variables with minimum effort For this reason miniaturized assay systems using 96-well plate assays were used Miniaturized assay systems are semiautomated with the use of automatic multi-well pipetters and ELISA plate readers to detect end points In addition to being able to handle a wide selection of variables, methods were simple to perform, involved no use of radioactive material and were sensitive as well as cost effective Acid phosphatase and crystal violet dye elution were both used as end points for miniaturized assay systems Angela Martin (Ph D thesis, 1992) compared the suitability of various end points used in miniaturized assay systems Acid phosphatase was presented as a suitable candidate for cytotoxicity and growth stimulatory assays (Martin and Clynes, 1991) For NRK cells, a linear relationship was found to exist between cell number and AP activity and the sensitivity was very good, it being possible to detect as few as 500 cells (Angela Martin, Ph D , 1992) However there was a loss of linearity at above $8x10^4$ cells per well, so it was important to ensure that cell growth did not exceed this upper limit

The other cell lines used in these investigations, MDCK and CHOK1 cells were analyzed for their ability to produce acid phosphatase A linear response for both cell lines was obtained when acid phosphatase production was compared to cell number

Acid phosphatase activity was however, merely a rapid screening method It was necessary to support the findings by looking at cell number to confirm the effects of specific variables This necessity was most evident from the results obtained in section 3 5 4 8 when investigating the growth promoting effects of the 0 5M NaCl fraction from heparin sepharose chromatography of BSA on NRK cells in low serum-supplemented medium Acid phosphatase production was found not to represent increased cell number in 96-well plates As crystal violet dye elution

was found to reflect changes in cell number, this was used as the end point for subsequent screening studies involving the 0 5M NaCl fraction

4.1 Development of low serum and serum-free medium for NRK cells

A number of SFM existed for NRK cells or NRK subclones, but attempts to grow the parental NRK cells in these SFM failed In order to develop a SFM, it was decided to investigate some of the more common serum-free additives in low serum conditions By using low serum concentrations, it was hoped to see what factors may be required in a SFM

Insulin, transferrin and albumin were studied and the optimum concentrations were found to be in the range used in most SFM (section 3 1 1) A lipid source has also been recommended for use in SFM (Glassy *et al* , 1988) A lipoprotein complex called Ex-cyte (Pentex product) was used as a lipid source Ex-cyte is a water soluble mixture of cholesterol and phospholipids which has been shown to support the growth of many lymphoid and hybridoma cells as well as fibroblasts such as CHOK1 and BHK21 cells (Hewlett *et al* , 1989) This was found to be most stimulatory at 20 - 30μg/ml The combination of BSA, insulin, transferrin and Ex-cyte was referred to as 'BITE' Many combinations like this appear in the literature *e g* HITES where H = hydrocortisone and S = selenium (Carney *et al* , 1984) BITE addition to 2% DHS was not as stimulatory as 5% FCS so a number of additional factors were tested

Without adding growth factors or hormones, it is possible to enhance biological activity by improving the basal media to provide additional nutrients usually supplied by serum In a series of experiments the effects of different basal media were investigated As cells were normally grown in DME, this was used as the basis for comparing all the basal media tested Where growth occurred, the addition of BITE improved the growth achieved by each basal medium The best growth was obtained using McCoys 5a basal media even though ATCC was the basal medium chosen for the SFM of NRK cells (Rizzino, 1984, Nugent *et al* , 1989, Newman *et al* , 1986)

The low growth response which occurred in Leibovitz-15 may have been due to the replacement of glucose as the energy source by L-galactose When glucose was present the level of amino acids correlated with the growth stimulatory ability McCoys 5a has a greater variety and in

general a higher concentration of amino acids than DME or Ham's F12 Further addition of non-essential amino acids to McCoys 5a medium did not result in improved growth as seen in section 3 1 6 Mitaka *et al* , (1991) when comparing the ability of basal media to support the growth of rat hepatocytes found that addition of amino acids, both essential and non-essential enhanced the growth in Leibovitz L-15 in comparison to ATCC

Of the inorganic salts and vitamins, the levels in ATCC were comparable to McCoys 5a, except that McCoys had more folic acid, myo-inositol, calcium and magnesium salts In addition McCoys 5a had vitamins not present in ATCC (nicotinic acid, ascorbic acid and aminobenzoic acid) ATCC had more inorganic salts ($CuSO_4$, $FeNO_3$, Fe_2SO_4, $MgCl_2$ and $ZnSO_4$) McCoys 5a is a very rich basal medium which has been used to support the growth of many primary cultured cells from normal bone marrow, skin, gingiva, testes, mouse, kidneys and lung and rat embryos (Hsu and Kellog, 1960) In looking at the growth of ovine satellite cells, Dodson *et al* (1990) compared several basal media (CRCM-30, low and high glucose DME, MCDB-104, McCoys 5a, Ham's F10 and M199) They found that use of McCoys 5a resulted in the best growth At 15% horse serum, McCoys 5a stimulated the best proliferation with up to 8 times the growth achieved by DME (high glucose)

However, McCoys 5a is not as defined as ATCC medium as it contains bactopeptone at a concentration of 0 6mg/ml Bactopeptone is a heat-stable enzymatic digest of animal tissues which is autoclavable and therefore not subject to possible viral or mycoplasma contamination More importantly, its precise composition is not known However, the fact that the cells remained viable in this basal medium was the basis for choosing it

At the same time as the ability of different basal media to support growth was investigated, a variety of growth factors and hormones were also tested as a means of improving the growth seen in 2% DHS and BITE (ATCC as basal medium) Two end points were used for analysis, acid phosphatase production and cell counts A combination of EGF, dexamethazone and BITE showed the best stimulation overall when AP (but not CC) was used as the end point (2 25 $\pm$ 0 61-fold stimulation above the control (2% DHS + BITE) for the three assays with CC as end point, 1 73 $\pm$ 0 72-fold stimulation for the three assays with AP as end point)

Dexamethazone alone showed increasing inhibition at increasing concentrations (more inhibitory with CC than AP) The combination of EGF and Dexamethazone was better than their additive

effects for AP and less than EGF alone for CC Dexamethazone has been reported to modulate the activity of EGF (by modifying EGF cell surface receptors qualitatively (Baker *et al* , 1978, Conover *et al* , 1989) At the same time, Dexamethazone has been found to be toxic for some cell types (Chilton *et al* , 1990)

With cell counts as the end point, best stimulation was seen with EGF, ß-Estradiol and BITE (2 04 $\pm$ 0 49-fold stimulation) ß-Estradiol showed no growth stimulation when used alone IL-1ß showed variable stimulation while IL-1α was inhibitory IL-1ß, together with TNF-α, has recently been reported to stimulate the production of mainly cytokine-induced neutrophil chemoattractant, equivalent to human gro/melanoma growth stimulatory activity in NRK-49F cells (Watanabe and Miyai, 1993)

Both α-FGF, heparin + BITE and ß-FGF + BITE were almost equally stimulatory at 10μg/ml FGF α-FGF and heparin were not stimulatory at 1ng/ml while ß-FGF was stimulatory at both concentrations Shipley *et al* , (1989) studied the effects of α- and ß-FGF on the growth of normal human keratinocytes and fibroblasts They found that for the fibroblasts, heparin (10μg/ml) improved the activity of α-FGF and the combination was almost as stimulatory as ß-FGF For the keratinocytes, α was better than ß and heparin slightly inhibited the action of α-FGF but dramatically inhibited ß-FGF activity

IGF-I did not show any stimulation alone or in combination with EGF This may be due to the presence of insulin which, for many cell lines, has been shown to exert its stimulatory activity by acting at the IGF-I receptors (MaGoffin and Erickson, 1988, Neely *et al* , 1991, McMorris *et al* , 1986) Insulin and EGF have been reported to act synergistically for NRK-49F cells (van Zoelen *et al*, 1985) As insulin activity in SFM often occurs through the IGF receptor, it was possible that the synergistic effect seen in section 3 1 3 was similar to that reported by van Zoelen However, if insulin and EGF were synergistic together, the stimulation caused by EGF and BITE would be expected to be higher

The combination of EGF and BITE was further studied in a lower serum concentration, 1% DHS In this situation, maximum stimulation for 3 assays together was 4 12 $\pm$ 0 24-fold stimulation over the growth achieved by BITE alone (EGF = 1ng/ml), while in an earlier experiment (Table 3 1 3 4) little growth was seen at 10 and 50ng/ml This combination of EGF and BITE was found to give better growth than that seen with 5% FCS using AP as the end

point (microscopic observation confirmed the extent of stimulatory activity with EGF) With the extent of activity seen for EGF+BITE and for McCoys 5a+BITE basal medium, it was decided to combine these results and eliminate the background serum Ex-cyte V was used originally and this was found to contain BSA fraction V As BSA was already in the combination, Ex-cyte III was chosen to replace Ex-cyte V However, Ex-cyte III was inhibitory so EGF (1ng/ml) replaced Ex-cyte as the 'E' in BITE

As no serum was being incorporated in the assay, an attachment factor called laminin was included (section 3 1 6) Again ATCC was found not to be as effective as McCoys 5a Addition of laminin did not improve growth However, Rizzino (1984) had found no requirement for fibronectin or laminin on plastic The most surprising result was that the BSA appeared inhibitory The BSA could be inhibitory due to interfering with the attachment of the cells or it may be that the concentration used, which was not toxic in serum-supplemented medium, had now become toxic

As MDCK and CHOK1 cells had been grown successfully in SFM it was decided to try the components of these SFM on the growth of NRK cells Variables were compared in ATCC (basal medium for MDCK SFM), Ham's F12 (basal medium for CHOK1 SFM) and McCoys 5a From the components m the MDCK SFM, insulin was most important From the components in the CHOK1 SFM, insulin and Fe_2SO_4 were most important Similar work was undertaken by Bradshaw and Dubes in 1983 They compared factors required by NRK-49F cells to those required by other kidney cell lines epithelial cells MDCK and LLC-PK_1 (pig kidney) and fibroblasts BHK-21 They found that the response of kidney cells varied The variations could have been due to species differences, origin of the cells (mesodermal as opposed to ectodermal) or even to the age or state of health of the animal when the cells were obtained Thus, finding that the SFM designed for the MDCK cells would not support the growth of NRK cells was not surprising

The extent of growth for the SFM over the basal medium was low This may have been due to some residual trypsin acting on the cells or the presence of some toxic compound which the cells could not deactivate or indeed to one of the components of the SFM So, trypsin inhibitor and EDTA were added to the SFM Trypsin inhibitor was added to inhibit the possible action of residual trypsin However, the omission of trypsin inhibitor did not affect the cells EDTA, which has been reported to act as a chelator of toxic factors (Bertheussen, 1993), showed

variable effects in three separate assays EDTA can have variable results depending on the metal to which it is complexed *e g* Hugenschmidt *et al* (1993), suggested that the tetrasodium or Cu-EDTA were found to be toxic to NRK-52E, while the Zn or Fe-EDTA were not)

Very surprising was the observation that omission of EGF resulted in a significant increase of growth, between 1 6-fold and 2-fold stimulation over the control (Figure 3 1 7 1) In low serum-supplemented medium EGF was perhaps the most stimulatory factor present EGF has been reported in the literature to be stimulatory to NRK clones in SFM (Nugent *et al* , 1989, Newman *et al* , 1986, Assoian *et al* , 1984) In combination with retmoic acid, anchorage-independent growth in soft agar was induced, but in monolayer growth was stimulatory (Jetten and Goldfarb, 1983)

In sections 3 1 5 and 3 1 6 in SFM with either ATCC or McCoys 5a, EGF showed only slight stimulation while in sections 3 1 7 1 and 3 1 7 3, EGF appeared to be inhibitory As the components in the SFM changed between the different sections and the original stock of EGF remained the same, it may have been possible for one of the components of the SFM to be modulating the activity of EGF In section 3 1 5, only insulin and transferrin were present in addition to EGF In sections 3 1 7 1 and 3 1 7 3, EDTA was additionally common to both It was possible that EDTA had in some way modulated the role of EGF, perhaps by binding divalent ions such as extracellular Ca^{2+} Many growth factors exert their mitogenic activity through a cascade of secondary messengers like 1,4,5 inositol tri-phosphate (IP_3) and diacylglycerol (Berridge, 1987) One of the results is the mobilization of Ca^{2+} which is largely due to Ca^{2+} release from intracellular stores but also from extracellular Ca^{2+} (Pandiella *et al* , 1987, 1988) While Ca^{2+}-channel blockers were reported to inhibit the action of recombinant PDGF on vascular smooth muscle cells (Block *et al* , 1989), EGTA only resulted in a reduction of mitogenic activity (Pandiella *et al* , 1987) The presence of EDTA may not account for the whole loss of activity associated with EGF

EGF has been reported to induce growth inhibition of A431 cells at concentrations greater than 1nM (6ng/ml), while being stimulatory at concentrations up to 0 1nM (0 6ng/ml) (Kawamoto *et al* , 1983b) This alternative effect was related to the presence of high and low affinity EGF-receptors It may be that EGF in our system was stimulatory in low serum-supplemented medium due to the up-regulation of EGF-receptors or alternatively, that growth in SFM resulted in a down regulation of high affinity EGF-receptors, which coupled to the presence or absence of EDTA may explain the variable loss of activity

The EGF receptor density is one of the major controlling parameters in density-dependent growth inhibition and phenotypic transformation of NRK cells. Up-regulation of the EGF receptor has been associated with the expression of the transformed phenotype in these cells when induced by TGF-ß, retinoic acid or PDGF (van Zoelen *et al.*, 1988). The level of EGF receptor expression in exponentially growing NRK cells was reported to be low (Rizzino *et al.*, 1990) and the level of binding decreased even further at high cell densities. So the activity seen in low serum-supplemented medium, may have been due to an up-regulation of the EGF receptor induced by some serum component (most likely TGF-ß or PDGF). However, anchorage-independent growth which occurs as part of the phenotypic transformation would have to have been inhibited in low serum-supplemented medium.

It is also possible that the loss of stimulation in SFM is due to the absence of an additional competence factor. For example, Olsen *et al.* (1988) found that EGF alone could not stimulate DNA synthesis in Swiss 3T3 mouse cells without the presence of brandykinin or prostaglandins. Nugent *et al.*(1989) found that EGF did not stimulate NRK cells alone but required TGF-ß for anchorage-independent growth, and that EGF was inhibitory to anchorage-dependent growth. Massague, (1985) had earlier suggested that TGF-ß induced a specific decline in the number of high affinity EGF-receptors in sparse monolayer cultures, thus reducing possible activity by EGF. As seen with the SFM for CHOK1 and MDCK, exclusion of insulin or Fe_2SO_4 separately, resulted in lower growth than that seen with the complete SFM.

So, using McCoys 5a supplemented with insulin, Fe_2SO_4 and EDTA as the basis, a variety of factors were screened for growth promoting activity (section 3.1.7). These included the trace elements used by Mendiaz *et al.* (1986), interleukins (IL-1, IL-4 and IL-6), phosphatidic and lysophosphatidic acid (van Corvan *et al.*, 1992; Bashir *et al.*, 1992), BSA and the 0.5M NaCl fraction from Heparin Sepharose chromatography of BSA, HDL (Rizzino, 1984) and growth factors (EGF, ß-FGF and PDGF) and Briclone. Of these factors the most stimulatory were found to be ß-FGF and PDGF. Variations in results were seen with IL-6, phosphatidic acid and the trace elements. Taub *et al.* (1979) noted different responses from the same component depending on the assay system used. The different activities of the factors could be explained by the variations made within the basic SFM for different assays. While serum-free Briclone was very stimulatory in one of the two assays, the entire composition of serum-free Briclone was unknown. As a result no further work was carried out using Briclone. Further analysis of serum-free Briclone components by fractionating may yield interesting results.

All these assays had been carried out using acid phosphatase as the end point The combination of ß-FGF and PDGF was then tested in comparison to the basal medium alone, and basal medium supplemented with 1% DHS and 5% DHS The results showed that while growth was significantly (20-fold) greater in the SFM than in the basal medium, the combination of ß-FGF and PDGF was 3 times less stimulatory than 1% DHS and 10-times less stimulatory than 5% DHS This showed that there were still factors to be found which would improve the growth in SFM

To confirm that the growth observed was due to the SFM and not to residual serum factors, it was necessary to subculture NRK cells in SFM (section 3 1 9) Results show that there was an initial lag phase, after which better growth was observed When after subculture 7, cells were no longer fed on day 4 of an 8 day incubation, much better growth was observed with the SFM containing ß-FGF + PDGF but not with the SFM containing ß-FGF (both SFM containing insulin and Fe_2SO_4) This could have been due to the cells taking time to adapt to the medium after passaging or it may be that the NRK cells were producing an autocrine factor which was only becoming active after a certain concentration had been reached It was also noted that in the presence of ß-FGF and PDGF, much fewer cells appeared to be floating in the suspension than when only ß-FGF was present

Comparing the SFM developed here with those reported for NRK cells and their subclones, small variations between SFM were seen Bradshaw and Dubes (1983) found FGF (50ng/ml) and PDGF (0 5 - 1 0U/ml) not to have any effect on NRK 49F cells, while insulin and retinoic acid did The SFM designed by Rizzino (1984) contained insulin, transferrin, FGF, laminin and HDL Lammin was found to have no effect on cell attachment in plastic which was used here Transferrin was not necessary as the SFM appeared to be better when Fe_2SO_4 was used (section 3 1 6 2 1) The level of FGF used by Rizzmo (1984) was much higher (1ng/ml as opposed to 150ng/ml) HDL had been found to be very slightly stimulatory at low concentrations and increasingly inhibitory at higher concentrations HDL was found to be inhibitory at 0 5µg/ml (as based on cholesterol concentration) while Rizzmo used 300µg/ml HDL The basis of determining the concentration may account for some of the difference between the levels used but could not account for such a big difference The HDL used in these studies was bovine-derived and contained 10µg/ml cholesterol and between 10 - 35µg/ml protein Cholesterol measurement was used as the basis for testing as the protein content was not known exactly. Rizzino had found the HDL not to be stimulatory alone but to show a

synergistic effect with the other factors In the present study, HDL was not tested with the other factors except insulin, ferrous sulphate and EDTA (section 3 1 7 4) In addition, the presence of bactopeptone in the medium may have reduced the requirements for lipoproteins

Production of autocrine activity

Bradshaw and Dubes found that growth of NRK-49F cells in SFM at low cell density was markedly increased by adding SFM that had been conditioned by NRK-49F cells at higher cell densities Without this conditioned medium, the cell growth rate at low densities was high through the first and second passages but subsequently decreased In the subculture described in section 3 1 9, cell growth decreased in passages 2 and 3 but appeared to be reaching a steady rate at passages 4 - 6 and again at passage 9 and onwards

Cells grown at high cell densities showed a similar rate of growth to that achieved with 10% FBS (Bradshaw and Dubes, 1983) At low cell densities (3,100 cells/cm^2), after 4 days with conditioned medium (CM obtained from high cell densities after 4 days growth), cell number was 4-times greater than that achieved without CM (Bradshaw and Dubes, 1983) The cells, in the SFM developed here, grew very slowly at first with very little observable growth after 4 days Thereafter the growth improved rapidly when the cells were not fed with fresh medium Rizzino also found that CM prepared from high density serum-free NRK-49F cells did stimulate the growth of NRK cells at low densities

In section 3 1 9, it is shown that leaving the medium on the cells for 8 days resulted in a doubling of the cell number for the SFM incorporating FGF and PDGF but not for the SFM with FGF The cell density was $3x10^4$ cells/cm^2 In comparison to Bradshaw and Dubes (1983) this is a low cell initial cell density A comparison of the initial cell density and the cell yield for the SFM is shown

Table 4 1 Comparison of cell yields for NRK cells in SFM

SFM	INITIAL CELL DENSITY	FINAL CELL DENSITY Cells/well	FOLD INCREASE IN CELL NUMBER	IT (Days)	REFS
Lm + FGF + Ins + Tf	10^5/35mm dish	4 2x10^5	4 2	3	Rizzino (1984)
Lm + Ins + Tf + EGF + FGF	3x10^4/35mm dish	9 0x10^4	3 0	3	Rizzino (1984)
	10^3 cells/ml 3 1x10^4/cm^2	4 7x10^4	4 7	4	Bradshaw (1983)
Ins + Fe_2SO_4 + FGF	3x10^4/35mm dish	19 46x10^4	6 4	8	Section 3 1 9 2
Ins + Fe_2SO_4 + FGF + PDGF	3x10^4/35mm dish	37 93x10^4	12 6	8	Section 3 1 9 2

Abbreviations Ins = insulin, Tf = transferrin, LM = laminin, IT = Incubation time

These results would suggest that PDGF was stimulating the production of some autocrine factor That PDGF stimulates the cells to grow and produce an autocrine factor would reflect the *in vivo* situation where PDGF is released during coagulation of blood and stimulates would healing *via* fresh growth of fibroblasts (Ross *et al* , 1986) Indeed, NRK-49F cells have been reported to produce a neutrophil chemoattractant equivalent to human gro/melanoma growth factor after induction with cytokines (Watanabe and Miyai, 1993)

Autocrine production induced by PDGF has been reported in human foreskin fibroblasts (Paulsson *et al* , 1987) where PDGF stimulated a transient (2 - 4 hours) autocrine production of more PDGF Human fibroblasts were also reported to produce somatomedin-like substances in response to PDGF (Clemmons, 1984) The autocrine factor remains to be identified

These results show that we have developed a SFM for NRK cells, with the only animal-derived protein being insulin and the small amount of BSA used to stabilise PDGF Fe_2SO_4 replaced the requirement for transferrin and the presence of recombinant ß-FGF and PDGF made this a low protein/serum-free media Although the supplements were well defined, the presence of bactopeptone in McCoys 5a removed the defined nature of the serum-free medium The SFM described here has only 10 006µg/ml protein not including the basal medium, but the basal medium contains 0 6mg/ml peptone

To obtain a truly defined medium, it would be necessary to replace the peptone with a combination of amino acids and/or peptides Alternatively, as Glasgows minimal essential medium (GME) was the next best basal medium to McCoys 5a, it may be possible to replace McCoys 5a with this as it is a defined basal medium In comparison to McCoys 5a, GME has a lower concentration of many vitamins and contains no ascorbic acid, biotin, nicotinic acid or vitamin B_{12} Supplementation with these vitamins together with additional essential and non essential amino acids may allow growth of NRK cells in GME as the basal medium for a SFM similar to that seen in McCoys 5a

4.2 Replacement of transferrin by inorganic/organic iron sources using MDCK cells in SFM

To investigate the replacement of insulin and transferrin, perhaps the two most universal components in SFM, two cell lines for which SFM already existed were chosen These were Chinese Hamster Ovary (CHOK1) and Madin-Darby Canine Kidney (MDCK) cells For MDCK cells, a SFM initially described by Taub in 1979 has remained essentially unchanged MDCK cells showed a requirement for prostaglandin E_1 (PGE_1) and transferrin Where a SFM for CHOK1 cells is concerned, Ham's F12 was originally designed for these cells, but changes and additions have been made frequently CHOK1 cells showed little requirement for transferrin (Appendix E) even though it was still included in the SFM designed by Mendiaz (1986) They did however, have a requirement for insulin These two cell lines offered suitable environments in which to look at the replacement of insulin and transferrin, with a view to developing a SFM devoid of animal-derived products

In order to investigate the replacement of transferrin, it was necessary to establish whether MDCK cells grew in the SFM and how well they responded to both insulin and transferrin When a growth curve was used to compare the response of MDCK cells in SFM and in serum-supplemented medium (SSM), a longer lag phase was observed in the SFM (Figure 3 2 1 1) On day 5, for 2 of the 3 assays, the cell yield in the SSM was 4 to 5-times that achieved in SFM By day 8, the cell numbers were almost the same, however at this stage, cells in the SSM would already have peaked and be in the decline phase of the growth cycle In later assays and subcultures, the MDCK cells appeared to grow almost as well as cells in SSM In the subculture experiments, initial assays were set up in closed systems *i e* in non-vented 25cm^2 flasks Within hours of inoculating the cells into the SFM or basal medium alone (ATCC), the

medium became very basic and the cells did not attach This was also observed by Edel Murphy who worked with MDCK cells in this laboratory (Edel Murphy, M Sc , 1986) The fact that this happened only in non-vented flasks and occurred with the BM and SFM only in the presence of MDCK cells indicated that the MDCK cells somehow may have caused an increase in the pH in the absence of CO_2 The buffers may also have had a deleterious effect on these cell lines in the absence of CO_2 and serum (HEPES has been found to inhibit some cell types in SFM by increasing superoxide dismutase and catalase inhibitable activities (Bowman *et al* , 1985, Bell and Quinton, 1991)) This problem was circumvented by using vented 25cm^2 flasks

When subcultured over a period of 40 days and 9 passages, slight adaption was apparent No direct comparison was made at this stage with SSM, but cells in SFM appeared to reach confluency around the same time as cells in SSM However, Jefferson *et al* (1985) subcultured MDCK cells for 10 passages in SFM and compared the protein synthesis patterns in passages 1 and 10 They found that MDCK cells grown in a hormonally-defined medium exhibited significant changes in both the specific activity of proteins and the labelling of specific proteins associated with the cell membrane The changes increased with increased cell passage in SFM An increased methionine uptake was suggested to be due to increased protein synthesis So although the cells would appear not to require significant adaption to SFM when analysing cell number, protein synthesis patterns show otherwise indicating the limitations of looking only at one variable as the end point of experiments

Comparing the results to those obtained by Taub *et al* (1979), growth rates of 1 5 doublings a day for SFM and SSM were seen In the first growth curve, the values for the SFM were much lower with doublings of 0 97 per day (assays 1 and 3) but in the subcultures an average of 1 1 doublings per day were obtained This average was obtained from all the subculture data but did not include subcultures that had grown over 5 day periods For the basal medium and SSM, doublings/day of 0 284 and 1 80 were obtained respectively The growth seen here in SFM was only 60% that obtained by Taub *et al* (1979) in SFM This variation in growth may have been due to a number of reasons, use of bovine instead of human transferrin, purity of some components of SFM, purity of ultrapure H_2O

MDCK cells were shown to be responsive to insulin and transferrin in SFM (Figure 3 2 1 3 and 4) Stimulation by insulin started at 0 5μg/ml (little or no activity at 0 1μg/ml), reaching a maximum growth of 1 9-fold to 2 5-fold stimulation over the control (no insulin) at 5 -

10μg/ml Transferrin showed stimulation with a maximum stimulation of 1 37-fold to 4 08-fold over the control (no transferrin) at 5 - 20μg/ml Cells showed very little stimulation by either BSA fraction V or BSA fatty acid free in SFM at low concentrations (0 1 and 0 5mg/ml) and became increasingly inhibited at higher concentrations (Figure 3 2 1 5) BSA fraction V was more inhibitory than BSA fatty acid free

Taub *et al* (1979) looked at the concentration profile of all the factors in SFM For insulin, our results compare very well, maximum stimulation was similar, occurring at 5 - 10μg/ml while Taub found maximum stimulation at 2 5 - 7 5μg/ml For transferrin, the extent of stimulation obtained in our experiments was variable (1 38-fold to 4 08-fold stimulation over the control), with maximum stimulation at 5 - 20μg/ml, presumably affected by the iron content of the SFM and the oxidation state of the iron For Taub, stimulation of up to 6 5-fold was reported at 5 - 10μg/ml No mention was made as to the extent of iron saturation of the transferrin *i e* whether it was apo-, partially- or fully-saturated 'Highly purified' bovine and human transferrin were compared to the insulin and transferrin normally used by Taub and were found to give similar results

In a hormone deletion study (Figure 3, Taub *et al* , 1979), omission of PGE_1 or transferrin was found to have the most serious effects on growth Omission of transferrin resulted in only 11% of the growth achieved by the complete SFM Omission of PGE_1 resulted in 50% growth while leaving out insulin only resulted in a 20% reduction in growth as compared to the complete SFM When omission studies were carried out while subculturing the cells, it was found that over a 5 week period, loss of any one component did not drastically affect growth except for transferrin and PGE (Taub *et al* , 1979) The fact that MDCK cell growth was significantly stimulated by transferrin meant that MDCK cells could be used to investigate the replacement of transferrin by inorganic and organic iron sources

Human transferrin is a glycoprotein of about 79kDa with two binding sites for a single trivalent atom of iron (referred to as N and C termini) *In vivo*, transferrin is the main carrier of iron Transferrin carries out two functions in SFM It acts as a source and transporter of iron into the cell and it also acts as a detoxifying agent by binding other metals present in the medium The addition of iron could not equal the growth stimulating effect of transferrin, suggesting that transferrin had a growth promoting effect independent of its transport activity, probably as a progression factor (Mather and Sato, 1979)

Transferrin interaction with the transferrin receptor is the main method of iron transfer across the cell membrane Other methods with and without transferrin have been reported (Basset *et al* , 1986, Inman and Wessling-Resmck 1993, Wright *et al* , 1986, Sibille *et al* , 1982, Crowe and Morgan, 1992 and Thorstensen, 1988) If methods other than transferrin receptor mediated endocytosis were operating in MDCK cells, a variety of iron containing compounds might replace transferrin Such compounds have already been used to successfully replace transferrin for a number of other cell lines These include simple soluble iron salts like Fe_2SO_4 (Mendiaz *et al* , 1986), ferric citrate and ferric ammonium citrate (Amouric *et al* , 1984) Simpler iron salts are liable to become oxidized in the SFM Thus, more complex chelators like ferric salicylaldehyde isonicotinoyl hydrazone (Fe-SIH) and ferric pyridoxal isonicotmoyl hydrazone (Fe-PIH) (Laskey *et al* , 1988, Sanders and Cheung, 1988, Brock and Stevenson, 1987) are often required However, it should be noted that iron is not always stimulatory Iron was found to inhibit the hormone responsiveness of pituitary cells (Sato *et al* , 1991, Eby *et al* , 1993)

We set out initially to see how important the level of iron loading on the transferrin was for its biological effect Transferrin can be referred to as apo- (iron poor), partially-saturated (1 molecule of iron bound per molecule of transferrin) or fully-saturated/holo (fully-saturated carries 2 molecules of iron per molecule) transferrin If all iron saturation levels of transferrin acted equally well, it would indicate the importance of transferrin as a transporter of iron rather than just as a source

Young *et al* (1979) found no difference between apo- and holo transferrin for SV3T3 fibroblasts Mather and Sato (1979) reported that less iron was required in medium where transferrin was incorporated Eby *et al* (1992) found that MDCK cells had a dependence for transferrin While di-ferric was able to support growth at concentrations up to 50 - 100μg/ml, the apo- (iron poor) was inactive at concentrations of 0 05 to 100μg/ml in iron poor medium

When apo-, partially- and fully-saturated transferrins were compared in SFM (section 3 3 1), all showed equally good stimulation at concentrations of 5 - 10μg/ml At higher concentrations, the apo- and partially-saturated transferrins became less stimulatory or inhibitory while the fully-saturated transferrin continued to be stimulatory This would coincide with the apo- and partially-saturated transferrins being at such high concentrations that they were scavenging the iron in the medium and making it less available to the cells in a transferrin-independent delivery system The fact that no inhibition was seen by Eby *et al* (1992) would make sense as the

medium was iron poor, so an inhibitory effect could not be seen as there was no additional iron for the apo-transferrin to chelate from the medium Perez-Infante and Mather (1982) also found apo- but not diferric transferrin to be inhibitory at higher cell concentrations If the inhibitory effect was due to the apo- and partially-saturated transferrin taking up iron from the medium then it would be expected that the apo- would be more inhibitory than the partially-saturated transferrin as it could bind two molecules of iron per molecule of transferrin, unless the rate of iron uptake by transferrin was higher for the second iron molecule than the first Studies of iron binding by Butterworth *et al* (1975) suggested cooperativity between the sites of ovotransferrin such that the affinity of the second site was increased by two orders of magnitude following binding of iron to the first site Additional information found that one of the two iron binding sites of serotransferrin was much more susceptible to the release of protons than the other Aisen *et al* (1978(a,b)) found this also, but the effect was found to be pH dependent While at pH 7 4, the affinity of the C-terminal site was 5-fold to 6-fold greater than the N-terminal, the difference at pH 6 7 was 20-fold This may thus account for the extent of inhibition seen by apo- and partially-saturated transferrin Alternatively, the apo- and partially-saturated transferrins may be binding other metals from the medium which could adversely affect metabolic and mitogenic activities

In choosing alternatives to transferrin the following components were assayed sodium mtroprusside (SNP), ferric nitrate (FN), iron choline citrate (ICC), ferric citrate (FC), ferric ammonium citrate (FAC), ferric ammonium sulphate (FAS) and ferrous sulphate (Fe_2SO_4) The results are shown in Table 4 2 1 which gives the ratio of maximum growth to the transferrin control for the average of three experiments The results show that the order of activity was $Fe_2SO_4 \geq$ Tf > SNP > FAS $\geq$ ICC > FAC $\geq$ FC > FN

Table 4.2.1 Growth stimulation of transferrin replacements in comparison to transferrin

Tf ALTERNATIVE	SNP	FN	ICC	FC	FAC	FAS	Fe_2SO_4
Average	0 902	0 657	0 885	0 672	0 673	0 888	1 049
$\pm$ standard deviation	0 367	0 086	0 107	0 129	0 168	0 075	0 300
Active range(μg/ml)	0 5 - 10 0	0 1 - 1 0	0 5 - 1 0	0 1 - 1 0	0 1 - 1 0	0 05 - 1 0	0 05 - 1 0

Results are expressed as the average growth relative to 5μg/ml transferrin $\pm$ standard error of the mean (n=3) Maximum stimulation in three separate experiments were taken to get the average

From these results, it appeared that Fe_2SO_4, FAS, ICC and SNP were almost as good as transferrin It was interesting to compare the replacements on the basis of the moles of iron

provided by each compound The order was Fe_2SO_4 > FC > FAC > SNP > FN > FAS > ICC This did not compare exactly with the order of stimulation although Fe_2SO_4 stimulated the best growth and had the highest iron content per mole of replacement of iron source After Fe_2SO_4, there was no correspondence between iron loading and the ability to stimulate growth The cause of this may be due to some of the compounds facilitating better uptake of iron than other compounds

Table 4 2.2 Transferrin replacement and iron loading per mole of replacement

Variable	SNP	FN	ICC	FC	FAC	FAS	Fe_2SO_4
Chemical Formula	$Na_2Fe(CN)_5NO$	$Fe(NO_3)_3\ 9H_2O$	$C_{33}H_{57}Fe_2N_3O_{24}$	$FeC_6H_5O_7$	$FeNH_4(C_6H\ O\ )_2$	$FeNH_4(SO_4)_2$	Fe_2SO_4
Mw	297 9	404 0	991 5	244 9	453 8	482 2	333 7
Moles per 1μg/ml solution (μM)	3 356	2 47	1 008	4 08	2 203	2 074	2 996
Moles of Fe per 1μg/ml solution (μM Fe/μM)	3 356	2 47	2 02	4 08	2 203	2 074	5 91

Note SNP contains 2 moles of H_2O per mole of SNP

To see if these factors could replace transferrin over a period of time, a subculture experiment was set up This would show if residual transferrin (which may have been internalized in the cells while setting up the assay) could be modulating the effects seen with the other iron complexes It would also show if there was a loss of the detoxifying ability of transferrin

It was also possible that the iron compounds did not act alone Bridges and Cudowicz (1984) found that the action of some iron chelators could exert a regulatory effect on the transferrin cycle in K562 cells They were found to enhance the release of apo-transferrin from the cell through the chelation of extracellular iron Chelators which could enter the cell were found to increase the number of new receptors being synthesised

Over a period of 9 passages in the first subculture, both SNP and FAS showed continued stimulation which was almost as good as that seen with transferrin Towards the end SNP was slightly better than transferrin Microscopic observations showed the cells in SFM supplemented with SNP and FAS instead of transferrin to look the same as those in SFM with transferrin The effect of SNP in neuroblastoma (M1) and glioma (C6) cells was suggested to be mediated by activation of guanylate cyclase which catalyses the generation of cGMP from

GTP (Zwiller *et al* , 1982) So SNP activity could be due to more than acting as a source of iron

ICC and Fe_2SO_4 did not maintain a high level of stimulation The cell yield was getting lower with each passage until at passage 8, there was not enough cells to continue on The cells in either of these SFM were ragged and the cytoplasm were spread out (in comparison to the Tf controls) So as seen with Fe_2SO_4 and ICC in the first experiment, good stimulation was not carried through to the subculture This may indicate that internalized transferrin (as transferrin bound to the transferrin receptor) may have been responsible for the transport of Fe_2SO_4 into the cells in the cell growth assay The dilution of remaining transferrin through several passages would result in less transport or iron and slower growth It was also possible that the state of oxidation of the iron complexes may have affected results as fresh samples had not been made up between growth assays and setting up the subculture experiment

Fresh samples of all iron compounds were prepared and a second subculture experiment was carried out Again, ICC was unable to support growth but Fe_2SO_4 was as stimulatory as transferrin (Table 3 3 3 2) However the experiment became contaminated after only the third passage The subculture was repeated (Table 3 3 3 3) The results supported the findings in the second subculture experiment ICC was unable to support growth It would appear that ICC had used residual transferrin in the growth stimulatory assays to facilitate transport of iron to the cells After only one passage, the growth was low, indicating the lack of a carrier molecule to effect growth Some cell lines have been reported to produce transferrin as an autocrine factor These include Reuber H-35 cells (Shapiro and Wagner, 1989), rat Rhodamine fibrosarcoma (Nagao and Nashikawa, 1989) and mammary epithelial cells (Lee *et al* , 1987) If autocrine production of transferrin were occurring for MDCK cells, subculture of MDCK cells in SFM without transferrin would not have died out within 5 passages as reported by Taub *et al* , (1979) In addition, the subculture in medium containing ICC would not have shown growth as low as was evident after several passages

It would appear that the oxidation state of Fe_2SO_4 affects its ability to stimulate growth The importance of the oxidation state of Fe_2SO_4 would necessitate making up fresh solutions on a regular basis

These results showed that transferrin could be successfully replaced in SFM with either FAS, Fe_2SO_4 or SNP FAS did not appear to suffer from a loss of growth stimulatory ability, while

SNP was not as consistently stimulatory FAS was not always the most stimulatory factor during the subculture experiments but it was found most consistently to give good growth and therefore, a reliable alternative to transferrin Growth rates similar to transferrin were maintained As PGE_1 can be replaced by Di-butyryl cAMP (Taub *et al* , 1984), the only source of animal-derived protein present in the SFM was bovine insulin Growth assays (Taub *et al* , 1979) showed that loss of insulin did not impede the cells from being subcultured in SFM IGF-I and recombinant insulin were tried as alternatives to bovine insulin but neither could support growth as well as bovine insulin However, the extent of stimulation by bovine insulin after 1 passage in SFM was minimal (Appendix D) So theoretically, MDCK cells could be grown in SFM devoid of any animal-derived proteins

4.3 CHOK1 growth in SFM and the role of Insulin

CHOK1 cells have great industrial importance due to their ease of transfection, rapid growth in suspension or on microcarriers and their biosafety status They have been used to produce many recombinant proteins Many SFM have been cited for the growth of CHO cells Ham's F12 was originally designed to support the clonal growth of such cells in a defined medium (Ham, 1965) However, without the presence of contaminating selenium, the medium was found not to support growth (Hamilton and Ham, 1977) Some of the SFM media designed for the growth of CHO cells include Hamilton and Ham, 1977, Mendiaz *et al* , 1986, and Darfler, 1990 MCDB was based on Ham's F12, but contains higher L-glutamine, $CaCl_2$ and lower cysteine In addition it contains 20 trace elements, including all the ones incorporated by Mendiaz *et al* (1986) The medium designed by Darfler is a complex combination of factors based on Ham's F12 containing in addition high concentrations of trace elements and a protein-free lipid emulsion

For these studies, the SFM developed by Mendiaz was used The growth response of CHOK1 cells in this medium was in the presence of fibronectin found to be much lower than that achieved with 5% FCS (Figure 3 2 2 1) Removal of fibronectin resulted in a dramatic improvement (Mendiaz found fibronectin failed to elicit a positive response) The growth in serum-supplemented medium (SSM) peaked at day 5 and then went into rapid decline while in SFM and basal medium, growth continued The decline in growth in SSM was probably due a combination of contact inhibition of the cells, accumulation of toxic waste compounds and a

lack of fresh nutrients Comparing SFM with and without fibronectin, on day 7, growth without fibronectin was 38 9 - 42x10^4 cells/well while in the presence of fibronectin only 6 - 11 4x10^4 cells/well was counted

Results obtained by Mendiaz showed growth in SFM to be 75% of that achieved in medium supplemented with 4% FCS after 4 days incubation The inoculation density used by Mendiaz was much higher than that used here (1 27x10^4 cells/cm^2 as opposed to 0 2x10^4 cells/cm^2), which may explain the low stimulation in these studies In the assay system, growth in SFM with and without fibronectin on day 3 (no day 4 for SFM without fibronectin) of 2-3% and 18-19% (in 2 assays) growth of the SSM was obtained respectively In a later experiment (section 3 2 2 6), this went up to 32% growth in SFM as compared with the SSM The differences in stimulation in these experiments were probably due to a combination of factors including the later incorporation of trace elements, improved user serum-free techniques, the purity of the components in the SFM and the ultrapure H_2O used to make up the basal medium

Calibration of cell number against acid phosphatase (AP) production showed a linear relationship in the range tested This end point was then used as a basis to investigate the response of CHOK1 cells in SFM to insulin, transferrin and albumin Insulin showed variable stimulation Two early experiments showed a maximum of 3-fold to 5-fold stimulation over the control (no insulin) while in two later experiments, 7-fold to 9-fold stimulation was seen Moreover, in assays 1 and 2, the growth response to insulin was high even at the initial concentration (0 1μg/ml) while in assays 3 and 4 at 0 1μg/ml, growth was only 20% above the control (no insulin) At 0 5μg/ml, the stimulation had dramatically increased to almost maximum levels

As the exact same pretreatment procedure was used in all assays, the question was asked whether the low extent of stimulation could be related to the possibility of insulin-independent mutants becoming expressed in assays 1 and 2? This could support the results observed the reduced stimulation seen by insulin as well as the almost steady state stimulation across the concentration range tested In assays 3 and 4, the greater response to insulin and the concentration-dependent increase in stimulation at low levels of insulin would indicate the absence of insulin-independent mutants Extrapolating from Figure 2 in Mendiaz's paper (1986), growth stimulation at 0 1μg/ml and 10 - 20μg/ml were 3-fold and 4-fold over the control (no insulin) respectively The results obtained from assays 1 and 2 compare favourably with these (but not with the results in assays 3 and 4) Other causes could be due to changes in the levels of components in the SFM, deactivation of water soluble vitamin components on

storage, changes in the levels of trace elements, loss of biological activities of insulin on storage at -20°C with time The variation in growth response of CHOK1 cells is further discussed on page 340

In CHOK1 cells, transferrin was found to have no significant effect in the presence of Fe_2SO_4 (Figure 3 2 2 4) If either transferrin or Fe_2SO_4 was omitted, little change in stimulation was observed, but when both were removed there was a significant loss in growth of about 50% (Mendiaz *et al* , 1986)

Albumin, BSA fraction V and fatty acid free were both tested for their growth stimulatory activity on CHOK1 cells (Figure 3 2 2 5) While both showed little stimulation at 0 1 and 0 5mg/ml, higher concentrations resulted in increased inhibition (so much so that negative values were obtained with AP as the end point)

As mentioned before, the real test of a SFM is its ability to support growth through serial passages This becomes very important on an industrial level where growth over an extended period in SFM can result in a greater product yield The results of the initial subculture (Figure 3 2 2 6 1) showed the SFM to only support growth for 3 subcultures and to fall off during the fourth passage It was thought that this may have been due to omission of the trace elements used by Mendiaz to maintain batch to batch consistency

In a growth curve (Figure 3 2 2 6 2), addition of the trace elements (TE) showed better growth than SFM without TE So this was used as a basis for an additional subculture With TE in the SFM, the CHOK1 cells were subcultured for up to 6 passages (Figure 3 2 2 6 3) In the fifth passage, a large increase in cell number occurred In the sixth passage no cells attached The same observation was made for a later subculture in which CHOK1 cells were subcultured for 9 passages (big increase in cell number in passage 8 and no attachment in passage 9) Mendiaz made no mention of subculturmg

The SFM described by Gasser *et al* (1985) allowed CHO cells to be grown for up to 40 passages in 10 months This SFM consisted of MEMs/Ham's F12 supplemented with 10μg/ml transferrin, 80mIU/ml (2μg/ml) insulin, 10^{-7}M selenite and $5x10^{-6}$M Fe_2SO_4 For these cells, transferrin was found to be the principal growth promoting factor He also found the growth promoting effects of insulin to be very variable When active, insulin reached maximum

mitogenic activity at 2μg/ml (superphysiological concentrations) However, a different subclone of CHO cells was used by Gasser *et al* , (1985)

Aside from a lack of essential components which may be missing in a SFM, another possible reason for the inability of CHOK1 cells to grow in SFM over extended periods could be related to the development of insulin-independent mutants as mentioned by Mendiaz Mendiaz found that insulin-independent mutants grew faster than parental cells and while they were stimulated by insulin during the first 48 hours, insulin became inhibitory at high concentrations (500ng/ml) In the subcultures carried out in these investigations, the second last subculture resulted in a larger cell yield than previously seen If these were insulin-independent mutants which had just formed, subculturing them into SFM containing insulin would inhibit their growth To determine if this is the case, CHOK1 cells could be grown in SFM with insulin until a huge increase in cell number is obtained The cells would then be split into two flasks (one with and one without insulin) If the observations were due to the formation of insulin-independent mutants, only the cells in the SFM without insulin would grow Other factors may have affected the long-term growth of CHOK1 cells in SFM Recently Bridges *et al* (1993), found that the extracellular matrix was important for the long-term anchorage-dependent growth of CHOK1 cells It was found that, after 5 passages of cells grown in SFM with 1ng/ml insulin, cells lost their ability to attach to tissue culture flasks but if coated with fibronectin, cells would attach In addition, the authors found that increasing concentrations of 10, 100 and 1,000ng/ml of insulin resulted in the cells becoming less flattened and rounded up and lost their ability to attach If fibronectin was added, attachment would again improve
These results would suggest that after a certain number of passages and/or the concentration of insulin can affect the ability of CHOK1 cells to lay down an extracellular matrix

The dependence of the CHOK1 cells on insulin made it a suitable system to study the replacement of bovine insulm with either a recombinant IGF or recombinant insulin

Insulin is, in general one of the major components of SFM, however the use of msulm at microgram/ml levels which are superphysiological, has led to many investigations into the biological activity of msulin For many cell types, insulin was found only to exert a mitogemc effect at high concentrations because it was cross reacting with the IGF-I receptor For some closely related cell lines like chinese hamster lung cell lines CC139 and V79, msulm was found to act *via* the IGF-I receptor (Van Obberghen and Pouyssegur (1983), Leckett *et al* , 1993)

For a small number of cell lines, the insulin was found to be active at nanogram levels, *via* its own receptor (Nagarajan and Anderson, 1982)

Mendiaz *et al* (1986), found that CHOK1 cells showed a significant increase in growth rate at insulin concentrations of 1 to 5ng/ml Thereafter, the growth rate was more gradual and reached a saturation point at 10μg/ml In support of this, Mamounas *et al* (1989), found that insulin acted at its own receptor with a half maximum stimulation of 14ng/ml In addition, it was found that IGF-I had little or no effect on DNA synthesis until concentrations of 225ng/ml, while IGF-II showed some stimulation of DNA synthesis with a half maximum activity at 75ng/ml However, this occurred with cells that had been starved for 72 hours in SFM without insulin Cells starved for 10 days showed the same profile

From the results it was shown that IGF-I was not an effective mitogen for CHOK1 cells at the concentrations tested IGF-II could stimulate growth but not as significantly as the bovine insulin Due to the low activity of the IGFs, it did not appear that insulin was acting to exert such a mitogemc effect through the IGF receptor The maximum activity of insulin occurred at slightly higher than normal physiological concentrations (50 - 100ng/ml)

Recombinant human insulin is a another possible alternative to bovine insulin Bovine insulin was found to be active at 0 01IU - 0 02IU/ml (0 389 to 0 77μg/ml) When comparing other sources of insulin it was found that the order of activity was bovine > ovine > recombinant human > porcine > equine insulin So recombinant human insulin could be used as an alternative to bovme insulin even though it was not quite as stimulatory

Some unexpected results occurred during this investigation the shift in activity and the variability in growth stimulation, the presence of inhibition at higher concentrations, the fact that 10μg/ml bovine insulin control was not inhibitory and all other insulins tested at this concentration were (fresh recombinant insulin was tested later and found not to be inhibitory at this concentration), there was also a distinct difference in the morphological observations made by Mendiaz *et al* , (1986) The shift in the maximum active range may possibly be explained by the increased sensitivity of the cells to insulin after subculturing in SFM before assaying

Of primary concern was the conflicting results showing bovine and the other insulins to be inhibitory at higher concentrations while the insulin control (10μg/ml) showed good stimulation in section 3 4 1 and 3 4 2 As seen in section 3 4 2, the higher concentrations of insulin showed decreased stimulation and eventually inhibition However, the control bovine insulin used as a control on each plate showed 3-fold stimulation over the control at 10μg/ml The control bovine insulin had the same maximum (or there abouts) stimulation as that for the insulin being tested All the other insulins were either not stimulatory or inhibitory at this concentration, possibly due to the following reasons

1 This discrepancy was not due to a pH effect For all insulins (except ovine insulin), initial reconstitution involved the use of 1N HCl (1N NaOH for ovine insulin) which may have affected the pH and so the growth of the cells Using the highest concentration of each insulin, the pH was measured and all were in the range pH 7 35 to 7 44 That all insulins showed this trend at high concentrations could suggest that some inhibitory factor got into the medium when the insulins were being reconstituted

2 From earlier results it could not have been the insulins themselves, unless some inhibitory factor was present when the insulins were reconstituted However, later experiments with the bovine and recombinant insulins showed significant activity (up to 10-fold stimulation over the control) at all concentrations tested (no inhibition)

3 Initial dilutions could have incorporated an inhibitory factor into the 10X insulin stocks that were used to make up all further dilutions However, similar experiments were carried out using the same insulin stocks with MDCK cells in SFM and no dilution of an inhibitory factor was observed *i e* if some component other than msulin was so inhibitory to CHOK1 cells, it was likely that some inhibition at higher concentrations of insulin would be seen on the MDCK cells in SFM which had been exposed to the same samples of insulin However, it could also be argued that the inhibitory effect may have been cancelled out by the growth stimulatory effect of the insulin for MDCK cells, resulting in no significant effect, as was seen

4 Mendiaz also presented information on the existence of insulin-independent mutants, which could become predominant in the serum-free medium cited without the presence of insulin If insulin-independent mutants had formed, these would be present in the control without insulin and the extent of growth stimulation over the control would be very low This could have

occurred in section 3 2 2 3 and 3 4 2 The inhibition in section 3 4 2 may possibly then be explained by the insulin-independent mutants being inhibited by high concentrations of insulin

5 The stock of insulin could have been inhibitory Later studies with old and new batches showed this not to be the case

6 Error in dilution If there was an error in the dilution when reconstituting the insulin from which the control was obtained, this would explain the fact that growth was seen at 10μg/ml provided the dilutional error made the insulin solution 10-fold less than it actually was However, using the same insulin stock and a new insulin stock (section 3 4 3) similar growth stimulation was seen at 10μg/ml for both stocks

It may be that this problem is linked with the variations in the extent of activity Could this be due to insulin-independent mutants? As mentioned in 4 above, this is a possibility The same batch of insulin showed different growth stimulatory responses, so the variation cannot be ascribed to the batch The passage number of the cells was investigated Passages 11 to 13 were used in section 3 2 2 and passages 4 to 7 were used section 3 4 However, even within each section, differing extents of stimulation were seen Passage number 4 and 14 were assayed and while some slight difference in stimulation was evident, it was not sufficient to account for the large differences in stimulation observed

The fact that the ratio of maximum stimulation by the insulins relative to the bovine insulin control remained the same regardless of the extent of activity, suggested that the variation in activity may occur in the basal medium If the insulin control (10μg/ml bovine insulin) were used as the control, the extent of stimulation between assays would be similar, the only problem would be the inhibition seen in section 3 4 2 It may be that the presence of some additional factor is required for cell growth in the absence of insulin and the concentration of this factor may vary from one batch of basal medium to another *e g* trace element levels or deactivation of water soluble vitamins on storage This would make sense as different batches of Ham's F12 were used in sections 3 4 1-2 and 3 4 3-4

The variation was not due to the pretreatment as all cell cultures in section 3 4 were grown in SFM before assaying In section 3 2 2 3, cells were not grown in SFM before assaying and still this variation in activity was seen Gasser also reported variability in the growth response of his CHO cell line (1985)

Variations in the morphological observations were conflicting with those of Mendiaz (Appendix K) When CHOK1 cells were grown in these experiments, in serum-free medium with or without insulin, they appeared fibroblastic in nature Mendiaz also observed that the CHOK1 cells that they used were only fibroblastic like in the absence of insulin in serum-free medium Morphologically, they looked like the CHOK1 cells that Mendiaz grew in SFM without insulin When Mendiaz incorporated insulin into the medium the cells became round to cuboid in shape This only happened with the CHOK1 cells used in these studies when the cells were 90 to 100% Migration of cells and formation of 'balls of cells' occurred for these CHOK1 cells in a similar way to that described by Mendiaz This was only seen when the cells were grown for a long period of time (2 weeks) in SFM without feeding and which had been set up at an initially low cell density In serum-supplemented medium (5% FCS), cells grew similar that described by Mendiaz (1986) Again only when fully confluent did the cells round up In addition, they seemed to form into balls of cells

In conclusion, CHOK1 cells were found to grow in the SFM described by Mendiaz *et al* (1986) over a number of passages However continuous growth in the SFM was not seen These cells were shown to be responsive to insulin In attempting to replace insulin, IGFs were tried For many cell lines the mitogemc action of insulin has been reported to be due to the insulin acting at superphysiological concentrations at the IGF-I receptor The IGFs failed to elicit a significant response This indicated that perhaps insulin was acting through its own receptor Recombinant human insulin was found to stimulate growth of these cells but was not quite as stimulatory as bovine insulin However, it can be used to replace bovine insulin in SFM As transferrin was found to have no effect, this meant that transferrin was the only animal-derived protein in this SFM and could be potentially removed due to the little dependence of CHOK1 cells on transferrin

4.4.1 Growth stimulatory effect of BSA on NRK cells

While investigating the optimization of a low serum-supplemented medium for NRK cells, it was found that addition of BSA resulted in increased growth (section 3 1 1) As it was hoped to use reduced serum supplementation with increased numbers of growth stimulatory factors as a basis for developing a SFM, the possible use of BSA as a growth stimulatory factor would

present problems BSA is such a 'sticky' protein that it could be carrying factors other than the desired product, also of course, it is an animal-derived protein with the consequence of possible biological contamination

Since it was possible that the activity associated with BSA could be isolated from the protein (Melsert *et al* , 1989, Kan and Yamane, 1982, Tigyi and Miledi, 1992), it was possible that the active factor could be replaced by a synthetic analogue, thereby maintaining the defined nature of the SFM Alternatively, if the BSA was found to be required as a carrier to facilitate activity, synthetic carriers which contain hydrophobic pockets like BSA could be used, for example ß-cyclodextrins for lipids

As this work was initiated before a SFM had been developed for NRK cells, many of the investigations to identify the source of the activity were carried out in low serum-supplemented medium (1% DHS) When a SFM was finally developed, the results were surprising The BSA stimulated the uptake of exogenous thymidine into the cells in SFM, but in a growth assay over 4 days, increasing concentrations of BSA resulted in increased inhibition Why this should be so was unclear That factors may be stimulatory in the presence of serum and inhibitory in the absence of serum (as seen in section 3 1 with EGF) indicate the extreme inter-dependence of factors in a complex medium These results call into question the use of low and very low serum-supplemented media as a means of identifying possible factors required for growth in SFM

Not only were NRK cells inhibited by BSA in serum-free growth assays, but the 0 5M NaCl fraction (isolated by heparin sepharose chromatography), which showed as much stimulation as the unfractionated BSA in low serum-supplemented assays, was just as inhibitory in SFM CHOK1 cells also exhibited a similar trend (section 3 5 4 3) These results for CHOK1 cells did not agree with the slight stimulation that BSA provoked in SFM in section 3 2 The reason for this may lie in the fact that the BSA was diafiltered in the experiment described in section 3 5 4 While Nilausen (1978) found diafiltration to improve stimulation by BSA for chinese hamster (CHEF) cells in SFM by the removal of inhibitory compounds, in this case diafiltration appeared to remove the stimulatory activity For MDCK cells, lack of stimulation was seen with both the 0 5M NaCl fraction and with the BSA, but no significant inhibition was observed (section 3 5 4 3)

The assay conditions under which NRK cells were set up to look at exogenous thymidine uptake and cell growth were similar In the thymidine uptake assay (section 3 5 4 13), cells were allowed to attach in the SFM for 18 hours, before addition of the BSA or 0 5M NaCl fraction Addition of the 0 5M NaCl fraction was based on the original concentration of BSA *i e* if BSA were at 1mg/ml, then the active component of the 0 5M NaCl fraction would be at the concentration that would be present in 1mg/ml of the original BSA sample and was referred to as 1mg/ml When cell growth was being measured, the same procedure was used, except that after addition of the 0 5M NaCl fraction or BSA, the assay was incubated for a further 4 days in order to allow cell growth to occur (section 3 5 4 14)

Uptake of thymidine was measured from 4 to 24 hours after the initial addition of BSA or 0 5M NaCl fraction, whereas the growth assay was terminated at 4 days after BSA addition Why uptake of exogenous thymidine was stimulated but cell growth inhibited is not known If the presence of BSA resulted in steric hindrance to physical attachment of the cells after an initial round of cell division, the original BSA would be expected to be significantly more inhibitory than the 0 5M NaCl fraction as the 0 5M NaCl fraction contained only 3% of the protein in the untreated BSA So sometime between 24 hours and 4 days at the end of the growth assay, the BSA and 0 5M NaCl fraction became apparently inhibitory

That the 0 5M NaCl fraction was inhibitory to NRK and CHOK1 cells in SFM was surprising BSA has been found to be stimulatory to a wide variety of cells in SFM and was incorporated into the SFM designed for NRK-49F cells (Newman *et al* , 1986, Nugent *et al* , 1989) However, various batches or fractions of BSA have been reported to be inhibitory for some cell lines in SFM (McKiernan and Bavister, 1992, Barlian and Bols, 1991) In the experiments presented here, the 0 5M NaCl fraction was as inhibitory as the untreated BSA It was possible that the 0 5M NaCl fraction stimulated entry of cells into the S phase of the cell cycle, but that other factors needed for completion of the cycle were absent in the SFM In a low serum-supplemented medium, all the factors for growth would be present in the medium but in a SFM, components normally supplied by the serum would need to be synthesized by the cells themselves or supplied exogenously The lack of these components may have had a deleterious effect on continued cell growth These results meant that neither the BSA nor the 0 5M NaCl fraction would be incorporated into the SFM designed in section 3 1 for the growth of NRK cells

In order to identify the putative 'active' factor, various methods used to isolate the activity from BSA which have been reported in the literature were investigated As this work was initiated before a SFM had been developed for NRK cells, much of the investigations were carried out in low serum-supplemented medium

A comparison of the different end points used to assay the growth stimulatory effect of BSA on NRK cells yielded some interesting results relating not only to the types of albumin tested but to the end points used BSA fraction V was shown to be stimulatory using all end points, with a 2-fold stimulation over the control (2% DHS) for acid phosphatase (AP) and cell number (CN) as end points With image analysis (IA) as the end point, 6-fold to 10-fold stimulation over the control was obtained, yet when the dye was eluted off the same plate (dye elution DE), only a 2-fold stimulation was obtained The fact that IA used colony area as a means of detection would suggest that BSA promoted both cell growth (as seen with other end points) and cell spreading (as seen with image analysis) For HSA fraction V, all end points showed a 2-fold stimulation, including IA This would indicate that while BSA fraction V promotes growth and spreading of NRK cells in low serum-supplemented medium, HSA fraction V promotes growth but has no additional effect on cell spreading

In later studies while investigating the activity of the 0 5M NaCl fraction, variability was again seen in the results (section 3 5 4 4) However, the variability could not be attributed to cell spreading only Acid phosphatase was originally used while screening fractions from HS chromatography In Run 6, low activity was obtained using AP When the samples were re-screened using IA and dye elution, the stimulation obtained by both IA and DE was greater than that obtained with AP That IA showed greater stimulation may, as has been said above, be due to detecting a spreading effect in addition to growth stimulation However, that DE showed greater stimulation suggested that in some way, AP was not properly representing changes in cell growth When AP, DE and cell number were used as end points for a BSA growth assay in 24-well plates, the extent of stimulation measured by AP was not much different to that measured by cell number or DE This would have suggested that perhaps the seeding density of the cells in the 96-well plate assays was not suitable, yet when the growth stimulatory effect of BSA was compared on the basis of initial cell density, DE was found to be consistently better than AP The reason for this is unknown

Due to the variations in growth stimulation exhibited by HSA and BSA fraction V and fatty acid free albumins, it was possible that fatty acids or lipids may have been responsible for the activity

Initially a commercially available lipid-protein mixture called Ex-cyte (supplied by Pentex) was tested in combination with HSA-faf Ex-cyte products encompass a range of water-soluble cell culture nutrient supplements derived from the lipid fraction of adult bovine or human sera All Ex-cyte products consist of a mixture of lipoproteins, cholesterol, phospholipids and fatty acids A typical batch of Ex-cyte III (bovine-derived) solution contains 9·9mg/ml cholesterol, 0 07mg/ml triglycerides, 8 04mg/ml phospholipids, 9 6mg/ml fatty acids and 19 5mg/ml protein (Pentext, Growth Enhancement Media Supplement) It has been shown to support the growth and monoclonal antibody production of AHT-107 cells in SFM (Hewlett, 1991) and the growth of BHK-21 cells in reduced serum conditions (Smith *et al* , 1990) The Ex-cyte when tested on NRK cells alone was inhibitory (Appendix B) Although some slight stimulation was seen when combined with HSA-faf, the extent of stimulation could not overcome the inhibitory effect of the albumin alone High density lipoprotein (HDL) was found to have little or no effect on the growth of NRK cells in SFM (section 3 1 7 4) even though it was present in many of the SF media described for NRK-49F cells (Rizzino, 1984, Newman *et al* , 1986 and Nugent *et al* , 1989)

Lipid content was found to be responsible for the growth promoting effects of BSA on Yoshida sarcoma (Yamane *et al* , 1975), SV40 transformed cells (Rockwell *et al* , 1980), HeLa Cells (Gerschenson *et al* , 1967) and Chinese Hamster cells (Ham, 1963, Nilausen, 1978) On the basis of these reports, a variety of lipids were loaded onto the albumin (oleic acid, cholesterol, phosphatidylserine and phosphatidylcholine) (section 3 5 1 4) No stimulation by the lipids without albumin on their own was found, and when loaded onto albumin no significant increase in stimulation was seen This may be because the lipids were not actually stimulatory to the NRK cells without a carrier However, both Nilausen (1978) and Jager *et al* (1988) suggested that oleic acid was just as good alone as it was when loaded onto albumin

These results indicated that if fatty acids or lipids were responsible for the difference in activity between BSA and HSA fraction V and fatty acid free, then the type of lipid or the concentration may not have been suitable for NRK cells The levels of oleic acid and cholesterol used were not excessive with levels of 1 - 25μg/ml reported in serum-free medium for several myelomas

and hybridomas, Chinese Hamster fibroblasts and adult rat hepatocytes (Jager, 1988, Nilausen 1978, Miyazaki *et al* , 1991) This would suggest that either the wrong fatty acids were tested or the fatty acids did not complex properly with the albumin and as a result not delivered to the cell The results seen with oleic acid and HSA-faf suggest, however, that loading took place (section 3 5 1 4 3) Also the affinities of fatty acids for albumin (especially delipidated albumin) are so strong, having association constants in the order of 10^7 to 10^9 (Spector and Fletcher, 1978) for long-chain fatty acids, that it was unlikely that loading did not take place Use of HSA-faf instead of BSA-faf may also have affected the results as BSA binds fatty acids more tightly, but both exhibit a strong affinity for fatty acid binding

It is of course possible that fatty acids other than those tested may have been responsible for the activity Gerschensen *et al* (1967) found 20 4 (arachidomc acid) and 18 2 (linoleic acid) to be the most important fatty acids for the growth of HeLa cells However, different nutritional requirements appeared to exist for different cell types Jager *et al* (1988) found that oleic acid could replace linoleic acid for X63-Ag8 653 myeloma cell line While Nilausen (1978) also found oleic acid to be as active as linoleic acid on the growth of chinese hamster cells CHEF, oleic acid could not replace linoleic acid for another chinese hamster cell line, CHD-3 (Ham, 1963) Keratinocytes took up linoleic acid from solution in preference to oleic acid (Schurer *et al* , 1994) Linoleic acid was found not to have any significant effect on the growth of NRK cells in SFM (section 3 1 6) although the lipids may have had other activities not detected by the end points used here, for example contributing to the structural integrity of cell membranes (Smith *et al* , 1982)

Gerschensen *et al* (1967) and Miyazaki *et al* (1991) also looked at stimulation by fatty acids and phospholipids without albumin The extent of activity observed with phosphatidylethanolamine, phosphatidylcholine and cholesterol was low, reaching a maximum of only 30% stimulation above the control (Miyazaki *et al* , 1991) From the results obtained in section 3 5 1 5, little or no activity was seen with acid phosphatase (AP) but some slight stimulation was seen with image analysis (IA) as the end point The fact that the activity was low may indicate that combinations of lipids were required or that the stimulatory activity was due to some other factor lost or deactivated during removal of lipids

A number of other possibilities to explain the difference in growth between fraction V and fatty acid free albumins exist The charcoal treatment itself may have been responsible for the loss of stimulation between fraction V and fatty acid free albumins of both species As delipidation

using charcoal at low pH is a harsh method, the treatment may have destroyed the activity of some component other than fatty acids Chen (1967) found that charcoal treatment did not drastically affect the albumin molecule itself as determined by analytical ultrafiltration, optical rotary dispersion and the ability to bind fatty acids However, a recent report (Sen *et al* , 1991) suggests that a change in the intrinsic optical activity of albumin was due to a change in the secondary structure of the protein, which may in turn have affected the binding sites and thus the biological activity of BSA

Another cell line, SCC-9 (human squamous carcinoma of the tongue) had shown responsiveness to albumin (section 3 5 1 2) For SCC-9 cells, no clear differentiation was seen in stimulation between fraction V and fatty acid free albumins, regardless of the source of albumin, human or bovine (order of stimulation was BSA-faf > HSA fraction V = HSA-faf > BSA fraction V) That both human fraction V and fatty acid free induced similar stimulation suggested that lipids were not important for the SCC-9 stimulating activity

Batch to batch variation may have affected the results (*i e* the BSA fraction V may have been from a 'good' batch while the fatty acid free may have been from a 'bad' batch; where both 'good' and 'bad' refer to the ability of the albumin to stimulate growth of NRK cells) Inter-batch variability has been reported with BSA (McKiernan and Bavister, 1992, Tomooka *et al* , 1985) To find out if the differences in activity of the fraction V and fatty acid free albumins could be due differences in the batches from which they were derived, both human and bovine albumins for which a commercially available fatty acid free component was available were obtained and tested on NRK cells (section 3 5 2 1) As the serum in which the assays were being carried out was equine, equine serum albumin (ESA) fraction V and fatty acid free were also tested For both BSA and HSA, fatty acid free albumins showed lower growth stimulation than the fraction V from which they were derived For ESA, fraction V and the fatty acid free showed little stimulation For rat tracheal epithelial cells, Thomassen (1989) found similar results when comparing BSA fraction V (A4503) and BSA-faf (A6003), albumin of the type used here However, no mention was made of the batch number

The initial isolation procedure may also interfere with the interpretation of results as the BSA-faf and the fraction V used in section 3 5 1 1 were isolated by different means The three most common initial fractionation steps by which albumins are fractionated are cold alcohol precipitation, salt fractionation and heat-shock treatment The results showed that the salt

fractionated albumin had almost no activity The heat-shock fractionated BSA was twice as stimulatory as the cold alcohol precipitated BSA Thus while the BSA fraction Vs prepared by cold alcohol precipitation showed roughly the same stimulation, no claim can be made that heat-shock fractionated BSA will always be better at stimulating growth of NRK cells as only one sample of salt fractionated and heat-shock fractionated BSA were tested Barlian and Bols (1991) looked at the effect of the initial fractionation and the effect on the growth of salmon embryo and trout gonadal cells 3 of the 5 BSAs prepared by cold alcohol precipitation were stimulatory while no fraction V prepared by heat-shock or salt fractionation supported growth

In summary, the results from sections 3 5 1 and 2 showed the growth promoting activity of BSA on NRK cells in low serum medium to depend on the source, the batch, charcoal treatment and probably the initial isolation step Comparison of BSA and HSA fraction V and fatty acid free albumins showed that charcoal treatment resulted in a reduction or total loss of activity respectively Attempts to replace the possible factors lost in the charcoal treatment with a commercially available lipoprotein mixture or individual lipids failed to restore activity To determine if the activity lost during charcoal treatment was due to a loss of fatty acids or to inactivation of some other factor, it would be necessary to carry out the charcoal extraction on the BSA, identify the fatty acids and lipids removed, and combine the identified fatty acids with the charcoal treated albumin to see if activity would be restored

4.4.2 Extraction of activity from albumin with organic solvents

Organic solvent treatment of albumin has been used to extract active components such as fatty acids and other lipids like lysophosphatides (Tigyi and Miledi, (1991 and 1992)) Lysophosphatidic acid has been shown to stimulate ^{3}H thymidine incorporation into a number of cell lines including Rat-1 fibroblasts (van Corven *et al* ,1989), Human foreskin fibroblasts (Jalink *et al* ,1990) and other fibroblasts (Bell *et al* , 1979) Much evidence to date suggested that lysophosphatidic acid could be an intracellular messenger with a role in cell growth and motility, possibly acting through a G protein-coupled receptor (Durieux and Lynch, 1993) In trying to isolate the activity associated with albumin which activated chloride oscillatory currents in *Xenopus* Oocytes, Tigyi and Miledi (1992) carried out sequential extractions with a variety of organic solvents Lysophosphatides were found to be present on serum albumin but not plasma albumin, suggesting that these were factors picked up by albumin during the clotting process The activity of the isolated lysophosphatide was tested on neurite retraction

in PC pheochromocytoma cells in SFM and also on ^{3}H thymidine incorporation into Sp2-O-Ag14 myeloma cells in SFM, where it was inhibitory

In an effort to determine if the lysophosphatidates were responsible for the activity seen with BSA on NRK cells, the extraction procedure of Tigyi and Miledi (1991,1992) was used (section 3 5 3) As solvent extraction has also been reported to extract fatty acids, it was of interest to see if the recombination could restore activity to the albumin The organic solvents used were methanol, ethanol, acetone, chloroform and di-ethyl-ether (assays carried out in low serum medium)

In two or three separate extraction processes (depending on the organic solvent), most of the activity remained with the protein phase for each of the solvents Very little or no activity was isolated with the organic phase Only when acetone was used as the extracting solvent was increased activity seen with recombination of the organic and protein phase, while only one of the two extractions with chloroform showed increased activity on recombination

The inability of recombination of the organic and protem phase to effect a recovery in activity would indicate that the active factor was not due to phosphatidic or lysophosphatidic acid The variable loss in activity in the protem phase may have been partially as a result of the process Extraction with organic solvents is a harsh treatment and may have resulted in the inactivation of some of the factors associated with the albumin It is also possible that some protein was lost during extraction process for example, as a result of protem residues sticking to gilson tips The results obtained by Tigyi and Miledi (1992) for the extraction of activity with the organic solvents did show some reduction in activity for DEE, chloroform and acetone for the protem phase, with no significant activity in the organic phase alone Only for methanol and to a lesser extent, ethanol was activity seen in the organic phase (Tigyi and Miledi, 1992)

To see if the vigorous mixing affected the activity of BSA, a control BSA dissolved into ATCC medium was exposed to 3 x 1 hour mixing at room temperature In three of the four assays no growth at all occurred Very few cells attached, most were suspended in the medium, with no increase in cell number Why BSA which had been stirred vigorously caused such a response from the cells is unknown the albumin samples exposed to various solvents did not elicit this response It may be that the excessive stirring denatured the BSA and exposed it to some component in the basal medium which hindered proper refolding of the albumin molecule

It also possible that toxic lipids were released As shown in Table 4 4 2, there was a high number of short-cham fatty acids present on the albumin and as short-chain fatty acids have been found in general to be inhibitory to cell growth (Ashbroke *et al* , 1975) the total loss of growth may be related to this

Tigyi and Miledi (1992) found that greater than 99% of the activity was removed after three washes in methanol However, for NRK cells, variable activity was retained in the protein phase (33 - 40% in the first, 82 - 84% in the second extraction and 88 5% - 106 9 the third extraction) In addition, Tigyi *et al* (1991) found the activity was abolished by trypsin digestion and slightly reduced by charcoal treatment Trypsin digestion by Tigyi and Miledi (1991) resulted in 27% of the activity seen with the BSA control In the results obtained in section 3 5 4 6, trypsin digestion carried out on the BSA resulted in no loss of activity in comparison to the BSA control, however the control in which trypsin inhibitor was added alone to the un-trypsinized BSA, showed increased stimulation over the BSA control alone If the trypsin digested sample was compared to this control (TI control), then 30 - 40% of the activity associated with the trypsin inhibitor control was retained

By addition of synthetic lysophosphatidates to inactive fatty acid free albumin, Tigyi and Miledi (1992) showed that activity could be restored Tigyi also suggested that as ethanol was able to solubilize the active factor, that albumins isolated with the Cohn procedure (Cohn *et al* ,1946) might have lost some activity This may explain why the heat-shocked albumin was found (section 3 5 2) to be more active than Cohn fraction V BSA
Commercially available lysophosphatidic and phosphatidic acid were added to the inactive unbound fraction from the heparin sepharose (HS) separation of BSA (see section 3 5 4) in an attempt to see if activity could be restored No activity was seen for LPA or PA either with or without the inactive albumin This is consistent with the finding that methanol extraction did not remove the majority of activity from BSA for NRK cells The extracted lipids (especially LPA or PA) could not account for the activity associated with the BSA For NRK cells in SFM, LPA and PA did not show significant consistent effect (Tables 3 1 7 2 3 and 3 1 7 3 1)
It is interesting to note that in addition to removal of lysophosphatidates, methanol extraction has been reported to result in a loss of fatty acids and neutral lipids While Tigyi and Miledi (1992) found only 30% of fatty acids were removed, Rosseneu-Motreff *et al* (1970), reported that methanol extraction removed 75% of fatty acids as well as phospholipids including lysophosphatidyl-sphingomyelin, phosphatidylcholine and phosphatidylethanolamine

In order to determine how effective the extraction process was and to quantify fatty acid content of the albumin in the first place, samples of untreated BSA, methanol and DEE extracted BSA were assayed for their lipid content The results are shown in Appendix F Lipid analysis of untreated and extracted albumins was carried out by Teagasc
It should be noted that the very small amounts of 'fat' extracted from the albumin meant that the determinations were made close to the limit of detection of the assay method

A study of the HPLC chromatograms of lipids extracted using DEE, methanol and no extraction (Appendix F), show that similar levels of C14 0, C16 0, C18 0, C18 1 and C18 2 were present Methanol-extracted BSA did not show the same trend as the other samples for the shorter chain fatty acids The amount of fat obtained from the methanol-extracted albumin was 10-fold higher than that obtained from the untreated or DEE-extracted albumin, however as seen from the chromatogram the levels of fats present in all samples appeared similar If this extracted amount of fat was correct, the lipid content of the BSA would have been 12 6% of the total sample dry weight This was most unlikely as BSA is normally sold with 96% or greater purity The purity of this particular batch of BSA was 96 4% (personal communication from Sigma)
Table 4 4 2 shows the moles of each fatty acid removed and the molar ratio of the fatty acids to albumin for the untreated and DEE-extracted BSA The methanol-extracted BSA could not be used due to the anomaly with initial weight of fat extracted from the albumin Overall, the lipid constituted 1 29% by weight of the untreated albumin and 1 13% of the DEE-extracted albumin The reduction in lipids on DEE extraction was only 12 4%

Table 4 4 2

VARIABLES	UNTREATED BSA		DEE-EXTRACTED BSA	
	μM in sample	Molar ratio to BSA	μM in sample	Molar ratio to BSA
BSA	6 786	---	5 635	---
C8 0	34 05	5 012	24 367	4 32
C10 0	0 24	0 035	0 15	0 027
C14 0	0 336	0 049	0 151	0 0268
C16 0	1 134	0 167	0 621	0 110
C18 0	0 498	0 073	0 318	0 056
C18 1	0 837	0 123	0 549	0 097
C18 2	0 569	0 0838	0 553	0 098

Results are expressed as the moles of fatty acids present in the sample

These results also show that for the higher chain fatty acids usually associated with growth (C16, C18, C18 1 and C18 2) there was little change with either extraction process and that the ratio of long-chain fatty acids to BSA was very low In addition, there was a high concentration of short-chain fatty acids which are not normally associated with growth

Average ratios of 1 or 2 moles of long-chain fatty acids per mole of albumin have been quoted Goodman (1957) found that charcoal treatment could reduce the molar ratio of fatty acids to albumin (HSA) from 1 82 to 0 02 The original fatty acid level reported by Goodman was over 10-fold higher than the levels of fatty acids detected in the untreated albumin in these studies The low initial levels of fatty acids may explain why the extraction did not remove many lipids

Addition of lysophosphatidic and phosphatidic acids to inactive BSA did not restore stimulation in low serum-supplemented medium Analysis of the untreated albumins and two of the extracted BSAs showed that low levels of long-chain fatty acids and higher amounts of short-chain fatty acids were present It may be that the activity of BSA was associated with a fatty acid other than that tested for or that had not been successfully removed from albumin, or that the activity was due to something entirely different

4.4.3 Heparin Sepharose Chromatography of BSA

Since albumin contains binding sites for a wide range of molecules in addition to lipids and fatty acids *e g* L-tryptophan (Brown and Shockley, 1982), steroids (Kragh-Hansen, 1981), Cu^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} (Peters, 1970), lysolecithin (Nilausen, 1968), ecosanoids (Unger, 1972), glucose (Shaklai *et al* , 1984) and folate (Soliman and Olesen, 1976), it is possible that the source of activity associated with albumin is other than fatty acids or lipids As it was not possible to define the active factor as a lipid, an alternative approach was taken Heparin sepharose was the most obvious of a number of affinity chromatographic techniques which could be used as a means of trying to isolate the activity

Heparin sepharose (HS) consists of heparin covalently coupled to a cross-linked sepharose matrix, Cl-6B It has been shown to bind a variety of proteins, including coagulation proteins, plasma proteins, lipases, lipoproteins, enzymes which act on nucleic acids, steroid receptors and protein synthesis factors (Handin and Cohen, 1976, Fujikawa *et al* , 1973, Kisiel and Davie, 1975) Due to its polyanionic nature, heparin interacts with many cationic compounds

The results using HS chromatography are shown in section 3 5 4 BSA was allowed to mix with HS overnight The mixture was then transferred to a column where the HS settled and the liquid was collected (unbound fraction) The HS column was washed with 50mM NaH_2PO_4 buffer (buffer wash) and further elution was carried out with step wise increases in salt concentration (0 5, 1 0 and 2 0M NaCl) The results for the protein concentration versus the activity for many of the fractionations are shown in Appendix G The results show that combining the unbound fraction and buffer wash, accounts for 97% of the protein recovered from the column No stimulatory activity and some inhibition was associated with these two fractions respectively The remainder of the detectable protein (about 3%), was found in the 0 5M NaCl fraction The activity of this fraction was found in all but one of 10 experiments to account for the majority of the activity associated with the untreated BSA The exception was Run 3, in which the activity appeared in the 1M and 2M NaCl washes The stimulation was found not to be due to higher salt concentrations (section 3 5 4 2) or to dramatic changes in the osmolarity (section 3 5 4 8)

As described in section 3 5 2, 'heat-shock' BSA was found to be more stimulatory than cold alcohol-precipitated BSA A sample of the 'heat-shock' BSA was applied to the heparin sepharose (HS) column, to see if the activity was isolated in the same fraction (section 3 5 4 18) As this was more stimulatory than the BSA initially used in earlier HS fractionations, it was hoped that the 'heat-shock' BSA would provide a better source from which to extract the activity Activity was eluted with 0 5M NaCl as seen with the original BSA (A4919), used but the extent of stimulation was equivalent to that obtained with this cold alcohol-precipitated BSA, indicating that more than one source of activity probably resided in the 'heat-shock' BSA Indeed, activity was also seen in the other fractions

As discussed earlier, the 0 5M NaCl fraction displayed all the activity associated with BSA The type of activity depended on the serum background, stimulatory in low serum and inhibitory in SFM In order to determine if the activity seen in the 0 5M NaCl fraction was due to a subfraction of albumin or a protein of a different molecular weight, samples of the 0 5M NaCl wash were run on SDS-PAGE under reducing conditions and stained with silver stain (section 3 5 4 5) The protein band in the 0 5M NaCl fraction migrated the same distance as the major albumin band No separate smaller bands were seen In addition no protein was detected in the 1M and 2M NaCl washes So, it was possible that the activity was due to a

subfraction of the albumin which specifically bound HS or the active component may have been bound very closely to albumin

If the activity was due to a protein, then digestion with either trypsin or pepsin could have resulted in loss of activity It was also possible that the protein present in the 0 5M NaCl wash was due to residual albumin which under more extensive washing with the buffer may have eluted off Trypsin digestion as monitored by SDS-PAGE (Figure 3 4 4 6) showed partial degradation in the first experiment and on extended exposure (18 hours), full degradation of the 0 5M NaCl band Biological activity of the 0 5M NaCl control was low in the first experiment after exposure to trypsin for 2 hours In the second experiment under the same conditions, addition of trypsin to the 0 5M NaCl fraction, either before or after addition of trypsin inhibitor resulted in a loss of stimulation while with the 0 5M NaCl fraction and trypsin inhibitor (TI) alone, growth stimulation above that caused by the 0 5M NaCl fraction was observed The activity of BSA did not appear to be affected by trypsm and it too showed increased stimulation when incubated with TI alone That the TI caused stimulation on its own presented problems in determining the extent of activity lost from the 0 5M NaCl fraction However, using either the TI control (0 5M NaCl fraction and TI) or the untreated 0 5M NaCl fraction as a control, the 0 5M NaCl fraction appeared to be partially sensitive to trypsin (varying between 23 4 - 44% and 44 - 69% activity retained depending on the control respectively)

Due to the problems with trypsm inhibitor, pepsin digestion was carried out in order to get a clearer picture (section 3 5 4 7) Again, no significant effect on BSA stimulatory activity was observed (about 20% lost) With the 0 5M NaCl fraction, only 10 - 22% of the activity was retained upon digestion with pepsin However, the pH control for this experiment, also showed low stimulation (15 7, 34 4 and 58 9% of the activity obtained for the 0 5M NaCl control for three separate assays respectively) Thus the total loss seen with pepsin was not due to pepsm alone but also to the pH The reduction in activity between the low pH and the low pH with pepsin varied between 35 and 68% in the three assays In a second pepsm digestion experiment, no difference in loss of stimulation was seen between the pH 2 6 sample with or without pepsin Later experiments which looked at the stability of BSA and the 0 5M NaCl fraction over a variety of pH (section 3 5 4 15), showed that BSA was relatively insensitive to extremes of pH For the 0 5M NaCl fraction, exposed to pH 2 6, variable activity was seen with 38 to 63% of the activity of the 0 5M NaCl fraction retained These results would indicate that exposure of the 0 5M NaCl fraction to pH 2 6 resulted in variable loss (as seen in two

separate experiments) in activity and that addition of pepsin could cause further inactivation in some cases This partial loss of activity due to exposure pH may have been caused, in part, to inadequate although vigorous mixing

Various analytical determinations were made (fatty acids, citrate and phospholipids) on the untreated BSA and 0 5M NaCl fraction (sections 3 5 4 9 - 11) However, the determinations were made at the detection limits of the diagnostic kits used As mentioned earlier, the biological activity associated with BSA is often attributed to fatty acids or lipids Kane, (1990) found that the activity associated with BSA that stimulated the growth of pre-implantation embryos was due to citrate The effect of citrate was examined on the growth of NRK cells A wide range of citrate concentrations, both with and without the unbound fraction were looked at, to see if activity could be generated However, no activity was seen

Following 24-hour dialysis against ATCC, 67 7 - 90 8% of the activity was retained (section 3 5 4 16) When compared using the ATCC dialysed control, only 35 - 57 6% of the activity was retained, which meant that some component of the basal medium (ATCC) was being lost resulting in lower growth in the ATCC alone Polet and Speiker-Polet (1975) found dialysis of BSA did not affect activity while Nilausen (1978) found increased activity after dialysis probably due to removal of inhibitory factors such as citrate, lactate, pyruvate and ferric ions (Hansen and Ballard, 1968)

The 0 5M NaCl fraction was reapplied to the column (section 3 5 4 17) The reapplication experiment showed that most of the detectable protein could be removed on reapplication of the 0 5M NaCl fraction The remaining protein migrated on an SDS-PAGE gel similarly to BSA It would be necessary to ascertain if the remaining albumin is part of the active component or a residual contamination from the separation process The 0 5M NaCl fraction was further studied using HPLC, gel filtration and diafiltration (discussed below)

4.4.4 HPLC of BSA and 0.5M NaCl fraction

An erythrotropin-like factor has been reported to be the active component associated with BSA for erythroid cells from fetal bovine liver (Congote, 1987) A C18 μBondapak column was used as the initial fractionation step in isolating erythrotropin-like factor from BSA by reverse phase HPLC The active factor had the same elution profile as fetal bovine serum erythrotropin

and elicited the same biological effect In order to determine if the activity associated with BSA for NRK cells could be due to an erythrotropin-like factor, BSA was fractionated using the procedure described by Congote As the column used in these studies was not a semi-preparative column, a larger number of runs were required to obtain sufficient volumes for assaying the bioactivity of the fractions on NRK cells

The protein elution profiles of BSA consistently showed 1 major peak at 50% Acetonitrile (ACN)) and three smaller peaks at the end of gradient at 75 - 80% ACN The major peak corresponded to the bulk of the albumin as seen in the lyophilized fractions, and bioassays showed this to be inhibitory Two areas of slight stimulation occurred, fraction 6 (before the main protein peak with no apparent protein peak (at 280nm) associated with the activity) and fraction 12 (corresponding to the first of the three smaller peaks) The activity in these fractions was low in comparison to the control BSA, being less than 20% and 37% of the control BSA, when dye elution and cell number were used as the end points respectively The 0 5M NaCl fraction was not very stable during lyophilization, which might indicate that the 'active factor' for NRK cells was not erythrotropin-like factor The protein elution profile showed the 0 5M NaCl fraction to elute off the column in the same place as albumin The three peaks at the end were found to exist as interference as they were present when no albumin was applied to the column

Coproporphyrin was used as a reference peak by Congote (1987) to determine the position of erythrotropin-like factor Samples of coproporphyrin were run to see if the small amount of activity had a similar retention time A sample containing BSA and coproporphyrin was applied to the column (Table 3 5 5 3 1) Over 5 runs, two peaks appeared consistently in the same positions The first of these peaks (peak 2) was at 31 5 - 32mm and the second peak occurred at 68 - 73mm and corresponded to the position of the main protein peak in these studies From these results, it was possible that the activity observed in fraction 6 could be related to erythrotropin-like factor However, Congote recovered 100% of the activity from the untreated albumin, while the activity here was very low The low activity may have been due to a separation of the erythrotropm-like factor and erythropoietin, with which it is very synergistic It may also have been that only a small amount of the activity was due to erythrotropm-like factor

The elution profiles obtained in these experiments were different to those obtained by Congote Congote's main protein peak eluted off very early at about 24 - 28% ACN while the main protein peak here eluted off about 50% ACN The second peak obtained by Congote (1987) corresponded to our first peak (50% ACN) Only a small peak was seen at 24 - 28% ACN When the albumin used by Congote (A8022) was run on the column, an elution profile similar to that seen with BSA (A4919) was obtained (Figures 3 5 5 5 1 and 2) The size of the column necessitated some changes in the running programme, however, as the separation was based on hydrophobic interactions it was very unlikely that the dimensions of the column would affect the acetomtrile concentration at which the albumin eluted off the column

The fact that Congote's first protein peak came off the column after such a short retention time suggested that the column may have been overloaded, which may have explained the differences in the protein elution profiles 2ml of a 40mg/ml solution was applied to Congotes' column at a time As the column used here was one quarter of the size of the column used by Congote, one quarter of the albumin should have been applied (20mg in total) in accordance with Congotes procedure (1987) However, the injection loop on the HPLC delivered a maximum volume of only 20μl With a maximum concentration of 200mg/ml albumin, only 4mg could be applied at a time Without a larger injection loop, it was not possible to prove whether overloading of Congote's column had taken place

A repeat experiment with the BSA and 0 5M NaCl fraction on the rp-HPLC resulted in variable growth inhibition in low serum assays Whatever the reason for the inhibition, further experiments involving studies on bioactivity were not to be depended upon However, the elution profiles of the protein, had not changed drastically during these experiments The elution profile of BSA-faf (A6003), BSA fraction V (A4919), BSA fraction V (A8022) and the 0 5M NaCl fraction were compared No significant changes in the elution position of the different albumins were observed (0 5M NaCl fraction contained a lot less protein in the mam peak)

It cannot be ruled out that erythrotropin-like factor is not present on the BSA used in these studies, however it did not appear to be the major active factor associated with BSA for NRK cells Congote, (1985) mentioned that fetal kidney cells were activated to a lesser extent than liver erythroid cells by erythrotropin Not only were differences observed in the response of different cell types but species variation in growth response to erythrotropin-like factor was also found between bovine and rat liver erythroid cells (Congote, 1987)

In order to further confirm that erythrotropin was not the major active factor, it was necessary to look at the molecular weight of the active factor in the 0 5M NaCl fraction The apparent molecular weight of erythrotropin-like factor is 9000 (Congote, 1987) If the molecular weight of the active fraction was not similar, then the factor being looked at was most unlikely to be erythrotropin-like factor To do this, the active fraction from the heparin sepharose fractionation was subjected to gel filtration

4.4.5 Gel filtration and Diafiltration of BSA and 0.5M NaCl fraction

Gel filtration was carried out to determine if any of the activity of the 0 5M NaCl fraction could be related to erythrotropin-like factor which has an apparent molecular weight of 9,000 When the 0 5M NaCl fraction was run on the column, three major protein peaks appeared (200,000, 130,000 and 66,000) in fractions that corresponded albumin polymers and monomers A number of other protein peaks were obtained (Table 3 5 6 2 3) Biological activity of the fractions were tested under low serum and serum-free conditions Samples were tested on the equivalent amount (mg/ml) that would be present in the untreated albumin

For low serum-supplemented media, activity was seen in fractions, 33 - 35, 54 - 60, 68 - 74 and 84 None of the fractions showed all the activity that would have been expected from the 0 5M NaCl control itself Activity reached about 50% stimulation over the control (1% DHS) This may have been due to the activity being eluted off over a number of fractions as seen in the bell-shaped curve of activity for some fractions It may also be due to the active factor requiring the BSA molecule to be present for activity

Comparing the position of activity with the protein profiles, the fractions 33 - 35 would have a molecular weight of 200,000 or greater which could indicate large polymers of BSA A protein peak in fraction 71 would correspond to the activity seen in fractions 68 - 74, which would indicate a molecular weight of about 2,400 If this were the major source of the activity associated with BSA, it would have to be tightly bound to albumin as diafiltration using a 3,000 molecular weight cut-off membrane could not remove it, yet for some reason it must have dissociated from albumin under the conditions used for gel filtration (this however, is unlikely)

For NRK cells in SFM, fractions exhibited no stimulation with the more complex SFM (insulin, Fe_2SO_4, ß-FGF and PDGF), while with the simpler SFM (insulin and Fe_2SO_4) some stimulation

was observed in fractions 79 - 81 and again in 84, which would indicate a molecular weight of 1,000 or less The extent of stimulation was very low

Gel filtration of the 0 5M NaCl fraction was repeated using low serum-supplemented assay system (section 3 5 6 3) However, the 0 5M NaCl control showed low stimulation (lower than had been seen previously with this same sample) The only major band of activity was isolated in fractions 74 - 79 which had a molecular weight of 2,000 or less This almost corresponded to the activity seen in the first gel filtration experiment No activity was seen in the fractions where the albumin eluted off the column

BSA was subjected to gel filtration in order to compare the results with the 0 5M NaCl fraction (section 3 5 6 4) In low serum-supplemented media, slight biological activity was seen with one of the BSA controls (which could invalidate one of the two assays) Activity was present in several fractions, 62 - 63, 85 - 87 and 88 - 100, but none of them were individually as good as the control BSA or as high as the stimulation seen with the 0 5M NaCl fraction As no activity was found at the point where the albumin eluted off the column, it would indicate that the albumin itself was not responsible for the activity, unless in some way the gel filtration procedure irreversibly destroyed the activity However, this is unlikely In addition, the spread of stimulation across a wide molecular weight band would suggest that a variety of factors were responsible for the activity Again, the possibility also existed that the active factor required BSA to exert its stimulatory effect

To investigate the possibility that recombination of factors with different molecular masses was necessary for activity, samples of BSA and the 0 5M NaCl fraction were diafiltered through membranes with molecular weight cut offs of 100,000, 30,000, 10,000 and 3,000

The results showed that for BSA virtually all of the activity was retained in 2 fractions (R_{100} and the $R_{30\text{-}100)}$, suggesting that the activity was due to monomers and polymers of albumin or to some factor bound to the albumin Following from the gel filtration, it would appear that the activity was due to some factor bound to albumin which although not removed during diafiltration, could become separated from the albumin molecule during gel filtration

No stimulation was caused by the $R_{3\ 10}$, the fraction which would contain erythrotropm-like factor, provided the factor was dissociated from the BSA during diafiltration Recombination

of the R_{3-10} with the R_{30-100} or R_{100} resulted in a small increase in activity in one experiment but not in the second, suggesting that some small activity may be found in the R_{3-10} However, as Congote (1987) used hydrophobic interactions to separate BSA and erythrotropin-like factor, it is possible that erythrotropin-like factor remained bound to the BSA during diafiltration

Diafiltration of the 0 5M NaCl fraction resulted in a different trend to that seen with the BSA None of the retentates showed growth stimulation individually Only on recombination of R_{100} + R_{10-30}, R_{30-100} + R_{10-30} and R_{100} + R_{10-30} was activity seen Of these, 60 - 80% of the activity of the 0 5M NaCl fraction was restored in the R_{10-30} + R_{30-100}, suggesting that the active factor had a molecular weight of between 10,000 and 30,000 daltons and required the presence of a small amount of albumin for activity However, this diafiltration was carried out only once

4.4.6 Summary on the activity associated with BSA on NRK cells

The results show that all of the growth stimulatory activity associated with BSA fraction V can be isolated in 3% or less of the original protein by means of chromatography using heparin sepharose The 0 5M NaCl fraction elicits all the stimulatory activity shown by BSA in low serum-supplemented medium and also the inhibition shown by BSA in SFM

Samples of the active fraction showed a band similar to that of BSA when run on SDS-PAGE gels This would suggest the presence of either a subpopulation of BSA or of BSA as a contaminant Reapplication of the 0 5M NaCl fraction to the heparin sepharose column, resulted in retained activity in the 0 5M NaCl fraction with a significant loss of protein The remaining protein showed the same migration as BSA on SDS-PAGE gels It is possible that the activity was due to a subpopulation of albumin Microheterogeneity of BSA has been widely reported (Kaplan and Foster, 1971, Janatova, 1974) Most commonly, fluctuations in the disulphide pairing on the albumin molecule, or the presence of bound impurities can result in heterogeneity The results obtained with HPLC and gel filtration would indicate that the activity was not due to the subfraction of the albumin molecule itself but rather to some impurity as the activity did not remain with the albumin, although of course, the putative active ligand may have been destroyed especially under the conditions used for rp-HPLC However, the possibility that this impurity binds to only a small amount of the albumin may indicate the presence of a subpopulation of albumin to which the impurity can bind

From lipid loading experiments and extraction of lipids from BSA, the activity would not appear to be due to fatty acids/lipids Reverse phase HPLC, gel filtration and diafiltration of BSA results would indicate that erythrotropin-like factor was not the major source of the activity

The activity of the 0 5M NaCl fraction was found to be partially susceptible to proteolytic degradation by trypsin or pepsin Diafiltration of the 0 5M NaCl fraction indicated that the activity was isolated in the R_{10-30} retentate and depended on the presence of a small amount of albumin for activity Further analysis of the more purified component (as isolated in the reapplication experiment), would yield more useful information as to the identity of the active component

In addition it was possible that the activity was not due to a growth stimulatory factor but rather the removal of some specific growth inhibitor from the basal medium or from the serum That the activity could be isolated using heparin sepharose would suggest a cationic nature for the active component Many factors have been isolated or purified using heparin sepharose chromatography including human platelet IV (Handin and Cohen, 1976), brain and pituitary fibroblast growth factor (Gospodarowicz *et al* , 1984), bovine factors VII (Kisiel and Davie, 1975) and IX (Fujikawa *et al* , 1973), apolipoprotein-H-like protein (Li *et al* , 1990) In addition to these factors, other impurities present on albumin include insulin inhibitors (Bajaj and Vallance-Owen, 1971), proteases (Zurawski *et al* , 1975) and nucleases (Anai *et al* , 1972) That albumin can carry such a broad variety of factors confirms how important it is for the source of the activity to isolated and defined for use in serum-free media

4.5 Implications of results for industrial applications

Although the investigations carried out in this thesis were based on the use of anchorage-dependent cells grown in monolayer cultures on a small scale, the results have implications which may apply to industrial situations

4.5.1 Development of defined media

The use of defined media offers potential advantages with respect to satisfying regulatory requirements and facilitating product purification (*i e* by reducing the amount of total protein in the culture system, purification of the desired protein can be made easier) To this end, the use of recombinant proteins and/or synthetic compounds provides an opportunity to replace animal-derived proteins normally used in SFM Three of the most commonly used factors used for small-scale and large-scale applications are the animal-derived proteins, bovine insulin, transferrin and albumin

From an industrial point of view, the CHOK1 cell line is probably the most important cell type of the three cell lines studied in these investigations These cells showed a high level of dependence on the presence of bovine insulin for growth stimulation monolayer SF cultures The possible use of recombinant IGF or recombinant insulin instead of bovine-derived insulin would remove an animal-derived protein from the formulation and in the case of recombinant IGF, reduce in the amount of protein present in the medium It was possible to replace bovine-derived insulin with recombinant human insulin, thereby providing a means by which CHOK1 cells could be grown in monolayer cultures in a SFM devoid of animal-derived proteins (the only other animal-derived protein in the SFM being bovine transferrin, which was found not to be necessary in the presence of Fe_2SO_4)

That recombinant human insulin could replace bovine-derived insulin for the growth of CHOK1 cells in monolayer, does not mean that all recombinant proteins or synthetic analogues can replace the ammal-derived components Indeed, it would be necessary to determine if the recombinant human insulin was a suitable replacement for large-scale applications of CHOK1 cells, grown either in free suspensions or on microcarriers Most importantly the productivity of the cells would have to be compared to ensure that the development of a more defined medium did not result in a drop of productivity or in alterations of the activity of the desired product Thomas and Fung (1994) found that a recombinant modified IGF-I (modified by truncation of part of the N-terminal which stopped the IGF from binding to IGF-binding

proteins) could replace bovine insulin for CHODXB11 cells without affecting the production of interleukin-1 or tumour necrosis factor receptors in a 2L serum-free fermentation

BSA is widely used as an additive in industrial-scale cell culture In this thesis, it was demonstrated that all of the growth stimulatory activity associated with BSA for NRK cells in low serum-supplemented medium was confined to about 3% of the BSA (*i e* the portion bound to heparin sepharose and removed by 0 5M NaCl) This confirms the necessity for assessing the role of BSA on growth and production As discussed earlier, in different systems BSA may act as just a carrier of the active factor (*e g* fatty acids, lipids or lysophosphatidic acid) which may replace albumin In situations where a carrier molecule is required, cyclodextrins and polyethylene glycol have been used Indeed, Blasey and Winzer (1989) were able to replace albumin with polyethylene glycol to allow growth of hybridomas in a low protein SFM where mAb production constituted a major part of the total protein

As the MDCK cell line is industrially important in the production of canine vaccines, the use of a SFM devoid of animal-derived proteins offers the same potential advantages as described for CHOK1 cells Bovine transferrin was successfully replaced by iron salts or iron complexes for MDCK cells grown in SFM On an industrial scale, the ability of iron complexes to replace transferrin (either human- or bovine-derived) will be affected by additional considerations Kovar (1990) suggested that the ability of insoluble iron complexes to replace transferrin for a number of hybridomas was due to physical contact when the complexes precipitate out of solution On scaling up, this form of contact would be less frequent and may reduce the effectiveness of the replacing factor Indeed, Metcalf (1994) reported that some iron complexes which could replace transferrin in monolayer culture were unable to replace transferrin in suspension cultures However Darfler (1990) found that soluble sodium nitroprusside could replace transferrin to allow protein-free culture of hybridoma cells with mAb production often exceeding 80% of the total protein while Cole *et al* (1987) could grow human-human hybridomas in a SFM but the viability and maximum cell densities were reduced in comparison to the albumin and transferrin containing controls

An alternative approach to that used in this thesis, would involve getting the cells themselves to produce the additional factors required to stimulate growth and obviate the requirement of adding animal-derived or recombinant proteins Kitano (1994) found that recombinant human

FGF could improve the growth of a human-human hybridoma, HBW-416 at low cell densities By introducing a plasmid containing the recombinant human FGF gene, a stable transformant was obtained which could be used to scale up to a 200L fermenter *via* two-steps from the master cell bank while the original cell line required 4 - 5 steps The transformant was able to produce good yields of the active antibody

It must be stressed that the use of one parameter *i e* growth (as used in this thesis), may not be a sufficient basis for deciding on the appropriate components or the concentration of components of a specific SFM, particularly when the aim is the large-scale culturing of cells to produce some factor other than biomass

4.5.2 Suspension versus anchorage-dependent growth and production

As mentioned above, differences may exist in the growth response and production rate of cells when grown in suspension cultures as opposed to anchorage-dependent growth Many anchorage-dependent cultures have been scaled up (by use of microcarriers and encapsulation for example) without a significant loss in productivity Reports by Kitano (1994) and Brand *et al* (1994), suggest that differences in growth and production may indeed be a problem, but one which is overcome by adapting the cells to grow in suspension and then reselecting for highly expressing clones Brand *et al* (1994) collated evidence to suggest that the specific production rate of recombinant CHO cell lines measured in static cultures in the presence of serum was a poor indicator of the subsequent performance of the cell line in SF suspension culture In this situation, the development of a high producing cell line was first obtained It was only after a high producer was isolated that it was subsequently exposed to serum-free conditions and suspension cultures simultaneously In this thesis, it was found that while albumin and EGF were stimulatory in low serum-supplemented media they had no effect or were inhibitory under serum-free conditions (depending on the concentration) As different sets of conditions result in differences in growth patterns, it may be necessary to optimise one parameter at a time *i e* selection of a high producing cell line in SFM and then adaption to suspension culture (Satoh *et al* , 1991)

Gawlitzek *et al* (1994) observed differences in the glycosylation of recombinant glycoproteins when comparing anchorage-dependent to anchorage-independent growth and serum-supplemented medium to SFM The results showed that BHK-21 cells grown in suspension cultures, secreted a higher proportion of N-glycosylated protein forms than cells grown on microcarriers However the effect of these alterations on the activity of the glycoprotein were not discussed

4.5.2 Prospects for use of SFM on industrial scale

Prospects for the use of SFM or protein-free media on an industrial scale will depend on the ability to overcome problems which result in the loss of serum including increased sensitivity to shearing effects, increased susceptibility to pH, increased susceptibility to proteolytic enzymes, reduction in productivity or alterations in the final product Many of these problems have been overcome by strict control of pH (Smiley *et al* , 1989, Gurhan and Ozdural, 1990), aeration and agitation (Marquis *et al* , 1989), addition of shear protective agents *e g* Pluronic F68 or methylcellulose (Papoutsakis, 1991, Marquis *et al* , 1989), reactor design *e g* perfusion cultures which control the growth of hybridomas to maintain a high viability while lengthening the phase of specific mAb production (Fazekas de St Groth, 1983) For some systems, the use of a SFM resulted in a higher productivity than in SSM (Satoh *et al* , 1991)

Problems relating to productivity on scaling up have in some cases been solved by the cloning and selection of more resilient cells In fact, the future use of SFM for large-scale applications may not so much depend on the development of a specific SFM tailored to a cell line but isolating a clonal cell line which is suitable to grow under pre-specified serum-free conditions and at the same time retain a high degree of productivity An example of this was shown by Kitano (1994) who adapted a human-human hybridoma (HBW-4 16) to a protein-free medium by use of repetitive cloning and selection The resulting cell line produced as much mAb as the original cell line By further exposing the cells to high shear stress in a spinner flask (by increasing agitation speeds), an improved cell line AGC-1 was developed which could produce 10 times as much IgG as the original cell line The AGC-1 cell line could easily be scaled up to 200L fermenter without loss of productivity

Other methods of increasing the productivity include increasing the gene copy number of the selected protein This selection of a highly-expressing system is carried out by the use of selective pressure, most commonly dhfr-MTX (Miyaji *et al* , 1990b, Satoh *et al* , 1991) or glutamine synthetase (Brand *et al* , 1994) Further improvements can be made by altering the gene product, *e g* Hosoi *et al* (1991) removed an AT-rich sequence in the 3' non-coding region of the ß-IFN gene This resulted in a more stable mRNA, which allowed for increased productivity Satoh *et al* (1991) removed a membrane binding region of M-CSF which increased the rate at which M-CSF was secreted

Alterations in the control of the growth cycle can also improve productivity Oh *et al* (1994) used high salt concentrations and the presence of butyrate to lengthen the stationary phase and enhance mAb production 2 3-fold over the hybridoma in the control medium Kessler *et al* (1994) found that growth in 'Ultraculture', a commercially available SFM could increase the stability of mouse hybridoma clonal cell lines by suppressing the rapid multiplication of non-secreting variants Growth in this medium resulted in increased chromosomal abnormalities, specifically in an increase in double minutes It was suggested that the stability of the clones also related to evolution of a hypertriploid-hypotetraploid karyotype

These trends indicate that the use of defined media conditions (low-protein and protein-free medium) are part of a concerted effort to make mammalian cell fermentations as economical and production as possible

CHAPTER FIVE

CONCLUSION AND FURTHER RESEARCH

5.0 CONCLUSIONS AND FURTHER RESEARCH

The aims of this thesis were to investigate alternatives to fetal bovine/calf serum in animal cell culture With respect to the three cell lines used in this project, SFM were already reported The efficiency of the SFM were to be investigated with special emphasis on the role of BSA, bovine insulin and bovine transferrin in the hope of replacing the animal-derived proteins with synthetic analogues, recombinant proteins or simple inorganic/organic salts This would result in upgraded SFM which would be devoid of animal-derived products and possibly low-protein or protein-free media

1 The investigations into the roles of BSA, insulin and transferrin for NRK cells were hampered due to the fact that NRK parental cells would not grow in the SFM designed by Rizzino (1984) for the subclone, NRK-49F It was therefore necessary to develop an alternative SFM As the cells would not remain viable in the basal medium (ATCC), a low serum-supplemented medium consisting of 1% DHS supplemented with 0 5mg/ml BSA, 5μg/ml insulin, 5μg/ml transferrin and 5ng/ml EGF was developed McCoys 5a was found to support greater cell growth than ATCC medium A SFM was subsequently developed using this basal medium supplemented with 10μg/ml insulin, 1 39μg/ml Fe_2SO_4, 5ng/ml PDGF and 1ng/ml ß-FGF This was found to support long-term (at least 10 passages) growth of NRK cells in a SFM

The apparently logical approach of examining the growth stimulatory effects of factors in the presence of low background of serum turned out to have serious limitations in that both EGF and BSA were stimulatory in the presence of serum and had no effect or were inhibitory (depending on the concentration) in SFM

While it was possible to get NRK cells to grow without serum supplementation, the SFM was not totally defined as it contained bactopeptone, bovine-derived insulin and BSA (to stabilize recombinant PDGF) To develop a completely defined medium, the following should be attempted

(a) Replacement of McCoys 5a with GME This was found to be the next best basal medium to McCoys 5a (section 3 1 4) As bactopeptone is a protein digest from meat extract, it may be possible to improve the growth in GME by further addition of ammo acids, both essential and non-essential

(b) Use of recombinant IGF-I or human insulin to replace bovine-derived insulin

(c) Use of cyclodextrins as a carrier for recombinant PDGF in place of BSA

2 As BSA fraction V was very stimulatory to NRK cells in low serum-supplemented assays, it was hoped to isolate the source of the activity, so that the BSA could be replaced by synthetic factors (*i e* specific factors *e g* fatty acids or analogues to albumin *e g* cyclodextrins), which could be used in SFM BSA fraction V strongly stimulated growth of NRK cells in low serum (2% and 1% DHS) supplemented medium, while BSA fatty acid free was not as stimulatory HSA fraction V was found to be growth stimulatory while HSA fatty acid free was mildly inhibitory To determine if the presence of lipids or fatty acids on the fraction V albumins was responsible for activity, a variety of lipids (oleic acid, cholesterol, phosphatidylserine and phosphatidylcholine) were loaded onto HSA fatty acid free Alone or in combination, the lipids failed to improve the ability of HSA fatty acid free to stimulate growth of NRK cells Also extraction of lipids from BSA fraction V with organic solvents failed to extract the majority of activity associated with BSA fraction V

Further investigations using heparin sepharose chromatography of BSA fraction V revealed that the activity associated with BSA was confined to less than 3% of the original protein and was consistently eluted with 0 5M NaCl The active factor stimulated growth to the same extent as the untreated BSA fraction V in low serum-supplemented medium However, in the SFM designed for NRK cells (section 3 1), the active factor was as inhibitory as the untreated BSA fraction V Further, the active factor and the untreated BSA fraction V were also found to be inhibitory with CHOK1 cells in SFM

Reverse phase-HPLC was employed to further identify the active factor However, a combination of exposure to acetomtrile, trifluoroacetic acid and lyophilization resulted in a loss of biological activity Further fractionation by gel filtration to determine the molecular mass of the active component, resulted in a loss of activity for the active factor and for BSA fraction V The loss of activity after rp-HPLC and gel filtration would suggest that the activity associated with BSA and the 0 5M NaCl fraction was not associated with an erythrotropin-like factor

Using diafiltration to find out if recombination of fractions were necessary to restore activity, diafiltration was carried out against a series of molecular weight membranes For BSA fraction V, all of the activity appeared to remain with the albumin protein (either as monomers or polymers) Thus, further work is required to the identify the component(s) of the active factor isolated in the 0 5M NaCl fraction obtained by heparin sepharose chromatography of BSA This may involve reapplication of the 0 5M NaCl fraction to heparin sepharose in order to produce a more purified active factor

3 For CHOK1 cells, which are industrially important, the aim was to upgrade the existing SFM to a defined medium free from any animal-derived proteins So the effects of bovine-derived insulin and transferrin were investigated The cells were found to be dependent on insulin but not on transferrin and as such was an ideal system to study the replacement of bovine-insulin It was found that bovine insulin could not be replaced by recombinant IGFs but could be replaced by recombinant human insulm It remains to be shown if the recombinant insulin can continue to replace bovine insulm over several passages in SFM Transferrin had little or no effect on growth and so could be removed from the medium By removal of transferrin and replacement of bovine with recombinant human insulin, a SFM devoid of animal-derived proteins could be prepared, the only protein present being recombinant human insulin In addition, as CHOK1 cells are industrially important, it would be necessary to look at their growth in SFM in large-scale systems, either in suspension or on microcarriers This would show if the replacement of bovine-derived insulin and transferrin were successful on scale-up As the yield of recombinant product is of prime importance on an industrial scale, it would be necessary to determine the effectiveness of the replacements on the yield of a desired product

4 For MDCK cells, the SFM designed for their growth contained bovine-derived insulin and transferrin As MDCK cells have been shown to be dependent on the presence of transferrin, and not very dependent on insulin, this was a suitable cell line for investigation of the replacement of transferrin in SFM with inorganic and organic complexes Of the factors which supported growth in the absence of transferrin, only some (ferric ammonium sulphate, ferrous sulphate and sodium nitroprusside) were able to support prolonged growth in serum-free, transferrin-free medium As insulin was the only remaining serum-derived protein (insulin was found not to exert a strong effect on MDCK cells) it may be possible to either remove insulin totally from the medium or replace it with recombinant insulin, thereby creating a SFM devoid of animal-derived proteins It is yet to be shown that the cells can continue to grow in a SFM totally devoid of animal-derived products over a period of time (several passages)

These investigations show that factors traditionally used in designing a SFM can be replaced by defined components which are not animal-derived There is as a result, great potential for developing totally defined low protein or protein-free media for a wide variety of cell lines

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APPENDICES

APPENDIX A

Appendix A details the contents of the basal media tested in section 3.1.5. The information was obtained from the respective catalogues (Gibco or Sigma). Values are given as mg/L.

Ingedients	DME	H F12	Mc 5a	GME	L-15	MEM	BME	RPMI
L-Alanine	—	9.00	13.36	—	225.0	8.90	—	—
L-Arginine.HCl	84.00	211.0	42.14	42.12	500.0	126.0	21.00	200.0
L-Asparagine	—	15.01	45.03	—	250.0	15.00	—	50.00
L-Aspartic Acid	—	13.30	19.97	—	—	13.30	—	20.00
L-Cysteine HCl	—	35.00	31.53	—	120.0	—	—	—
L-Cystine	62.60	—	—	30.22	—	31.30	15.65	65.20
L-Glutamic acid	—	14.70	22.07	—	—	14.70	—	20.00
L-Glutamine	58.40	146.0	219.2	584.6	300.0	—	292.0	300.0
Glutathione	—	—	0.50	—	—	—	—	1.00
Glycine	30.00	7.51	7.51	—	200.0	7.50	—	10.00
L-Histidine	42.00	20.96	20.96	—	250.0	42.00	8.00	15.00
L-Hydroxyproline	—	—	19.67	21.00	—	—	—	20.00
L-Isoleucine	105.0	3.94	39.36	52.46	125.0	52.00	26.00	50.00
L-Leucine	105.0	13.10	39.36	52.46	125.0	52.00	26.00	50.00
L-lysine	146.0	36.50	36.54	73.06	93.70	72.50	36.47	40.00
L-Methionine	30.00	4.48	14.92	14.92	75.00	15.00	7.50	15.00
L-Phenylalanine	66.00	4.96	16.52	33.02	125.0	32.00	16.50	15.00
L-Proline	—	3.45	17.27	—	—	11.50	—	20.00
L-Serine	42.00	10.50	26.28	—	200.0	10.50	—	30.00
L-Threonine	95.00	11.90	17.87	47.64	300.0	48.00	24.00	20.00
L-Tryptophan	16.00	2.04	3.06	8.16	20.00	10.00	4.00	5.00
L-Tyrosine disodium salt	103.8	7.78	22.51	45.0	372.8	51.90	25.95	28.83
L-Valine	94.00	11.70	17.57	46.86	100.0	46.00	23.50	20.00
Ascorbic Acid	—	—	0.50	—	—	—	—	—
Biotin	—	0.007	0.20	—	—	—	1.00	0.2
D-Ca pantothenate	4.00	0.48	0.20	2.00	1.00	1.00	1.00	0.25
Choline chloride	4.00	13.96	5.00	2.00	1.00	1.00	1.00	3.0
Folic acid	4.00	1.32	10.00	2.00	1.00	1.00	1.00	1.00
i-Inositol	7.20	18.00	36.00	4.00	2.00	2.00	2.00	35.00
Niacinamide	4.00	0.037	—	—	—	1.00	1.00	1.00
Nicotinamide	—	—	0.50	2.00	1.00	—	—	—
Pyridoxal HCl	4.00	0.062	0.50	2.00	—	1.00	1.00	—
Pyridoxine HCl	—	—	0.50	—	1.00	—	—	1.00
Riboflavin	0.40	0.038	0.02	0.20	0.1076	0.10	0.10	0.20
Thiamine HCl	0.40	0.034	0.02	2.00	1.00	1.00	1.00	1.00
Vitamin B_{12}	—	1.360	2.00	—	—	—	—	0.005

INGREDIENTS	DME	H F12	Mc 5a	GME	L 15	MEM	BME	RPMI
$CaCl_2$	265 0	0 002	132 5	264 9	185 5	265 0	265 0	---
Cupric sulfate $5H_2O$		0 002	---		---	---	---	---
Ferric Nitrate	0 100	--	---	0 10	-	---	---	---
Ferrous Sulfate	---	0 834	--			---	---	---
Magnesium Chloride $6H_2O$	---	123 0	-			---	---	---
Magnesium Sulphate	97 67	---	200 0	200 0	400 0	97 67	97 67	48 84
Potassium chloride	400 0	224 0	400 0	400 0	400 0	400 0	400 0	400 0
Sodium bicarbonate	---	---		--	---	2,200	2,200	---
Sodium Chloride	6,400	7,599	6,460	6,400	8,000	6,800	6,800	6,000
Sodium Phosphate monobasic	109 0	---	655 7	2,750	---	122 0	122 0	---
Sodium Phosphate Dibasic	---	142 0	---	- -	190 0	-	--	800 0
Succinic Acid		- -	---		- -	--	---	---
Succinic Acid (disodium $6H_2O$)	---	---	-		-	---	-	---
Zinc Sulfate $7H_2O$	---	0 086			- -	---	---	---
D-glucose	1000	1,802	3,000	4,500	- -	1,000	1,000	2,000
Hypoxanthine	---	4 08		---	---	---	--	---
Linoleic acid	---	0 084	- -	-	- -	---	---	---
Phenol Red	15 90	1 30	10 00	17 00	10 00	11 00	11 00	5 30
Putrescine HCl	---	0 161	---	---	---	-	---	---
Pyruvic Acid	110 0	110 0	---		---	-	---	---
Thioctic Acid		0 021	---		---	---	---	- -
Thymidine		0 073	---		-	---	---	---

APPENDIX B

Appendix B shows the effects of Ex-cyte III on the growth of NRK cells in low serum-supplemented medium (1% DHS) Results are expressed as the average percentage growth relative to control (1% DHS) Acid phosphatase was used as the end point for experiments Table B shows the results for three separate experiments

Table B Effect of Ex-cyte on NRK cells

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
1% DHS	100 0 ± 8 28	100 0 ± 8 89	100 0 ± 8 74
+ 2 5μg/ml Ex	91 39 ± 11 9	87 37 ± 4 89	92 52 ± 8 77
+ 5 0μg/ml Ex	83 94 ± 7 95	85 54 ± 7 54	94 29 ± 7 22
+ 10μg/ml Ex	81 62 ± 4 92	86 16 ± 5 40	94 80 ± 13 0
+ 20μg/ml Ex	75 07 ± 12 3	70 27 ± 6 20	76 42 ± 9 88

Abbreviations Ex = Excyte III Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8)

APPENDIX C

Appendix C shows the growth response of CHOK1 cells while being subcultured in serum-free media The SFM was designed by Mendiaz *et al* , (1986) Results are expressed as the cell Yield x 10^{-4}/flask $\pm$ standard deviation (n=3) The results for 3 separate experiments are shown in Table C

Table C Cell Yield x10^{-4}/flask

SUBCULTURE	ASSAY 1	ASSAY 2	ASSAY 3
1	177 2 $\pm$ 15 9	325 5 $\pm$ 30 3	330 0 $\pm$ 20 0
2	138 7 $\pm$ 0 14	145 0 $\pm$ 11 3	137 2 $\pm$ 4 58
3	102 4 $\pm$ 6387	147 7 $\pm$ 9 56	127 5 $\pm$ 11 5
4	128 6 $\pm$ 10 1	309 0 $\pm$ 13 8	124 9 $\pm$ 14 9
5	237 7 $\pm$ 32 9	296 2 $\pm$ 78 0	224 2 $\pm$ 6 36
6	61 00 $\pm$ 3 82	55 32 $\pm$ 7 22	34 84 $\pm$ 2 88
7	77 35 $\pm$ 0 36	32 74 $\pm$ 0 48	35 50 $\pm$ 7 64
8	150 5 $\pm$ 1 41	156 7 $\pm$ 11 7	268 5 $\pm$ 29 7

Subcultures were carried out every 4 days

APPENDIX D

Appendix D shows the growth response of MDCK cells to IGF-I and recombinant human insulin under serum-free conditions The results are expressed as the average percentage growth relative to control (no insulin) ± standard deviation (n=8) Acid phosphatase was used as the end point and the results for 3 separate experiments are shown in Tables Da and Db

Table Da IGF-I

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0nM IGF-I	100 0 ± 2 99	100 0 ± 6 59	100 0 ± 7 57
SFM con	109 5 ± 4 19	167 5 ± 3 88	108 7 ± 9 11
0 1nM IGF-I	86 73 ± 4 12	74 04 ± 3 14	102 0 ± 9 83
0 5nM IGF-I	86 31 ± 3 39	70 72 ± 3 69	94 77 ± 9 15
1 0nM IGF-I	86 99 ± 6 56	75 15 ± 2 58	92 28 ± 9 90
5 0nM IGF-I	----------	75 33 ± 4 43	77 88 ± 4 85
10 0nM IGF-I	72 81 ± 7 24	95 46 ± 8 08	69 92 ± 5 71
20 0nM IGF-I	79 11 ± 4 49	104 1 ± 9 72	88 70 ± 3 45
50 0nM IGF-I	70 60 ± 0 02	99 34 ± 8 86	108 7 ± 9 87

SFM con refers to the SFM designed by Taub *et al* , (1979) containing 5μg/ml bovine insulin

Table Db Recombinant human insulin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0IU/ml	100 0 ± 1 74	100 0 ± 3 55	100 0 ± 8 48
SFM con	111 6 ± 2 69	106 0 ± 6 22	100 9 ± 4 63
0 0002IU/ml	87 87 ± 3 06	82 80 ± 4 76	86 36 ± 6 26
0 001IU/ml	87 32 ± 3 15	75 60 ± 3 71	90 83 ± 5 43
0 002IU/ml	87 32 ± 4 29	75 00 ± 4 79	82 81 ± 4 38
0 01IU/ml	76 53 ± 3 86	77 77 ± 5 03	92 89 ± 7 85
0 02IU/ml	89 52 ± 2 22	79 49 ± 2 79	91 39 ± 8 97
0 10IU/ml	95 40 ± 3 67	88 88 ± 4 49	103 3 ± 7 18
0 20IU/ml	95 71 ± 4 47	79 63 ± 4 44	87 60 ± 5 63

SFM con refers to the SFM designed by Taub *et al* , (1979) containing 5μg/ml bovine insulin

APPENDIX E

Appendix E shows the growth response of CHOK1 cells to transferrin under serum-free conditions (SFM designed by Mendiaz *et al* , 1986) The results are expressed as the average percentage growth relative to control (no transferrin) ± standard deviation (n=8) Acid phosphatase was used as the end point and results for 3 separate experiments are shown in Table E

Table E Transferrin

VARIABLES	ASSAY 1	ASSAY 2	ASSAY 3
0 0μg/ml	100 0 ± 5 97	100 0 ± 5 51	100 0 ± 8 05
SFM con	96 55 ± 10 9	107 6 ± 11 3	78 49 ± 4 09
0 01μg/ml	98 76 ± 7 64	95 85 ± 10 3	88 74 ± 10 6
0 05μg/ml	101 9 ± 6 93	98 19 ± 8 47	93 03 ± 6 70
0 1μg/ml	99 44 ± 4 13	89 19 ± 6 31	84 15 ± 7 76
0 5μg/ml	105 7 ± 7 07	86 30 ± 7 39	94 19 ± 7 85
1 0μg/ml	107 6 ± 5 89	107 7 ± 7 75	91 71 ± 5 08
5 0μg/ml	100 4 ± 6 37	97 29 ± 9 01	83 40 ± 4 02
10 0μg/ml	-------	114 4 ± 7 93	81 96 ± 9 20
50 0μg/ml	87 26 ± 6 47	97 84 ± 6 49	101 7 ± 7 51

SFM refers to the SFM designed by Mendiaz *et al* (1986) containing 5μg/ml partially saturated bovine transferrin

APPENDIX F

Appendix F shows a report on the analysis of albumin samples for fatty acid content Due to the very small amount of fat extracted from samples, determinations were made close to limits of the method The analysis was carried out by Teagasc, The National Food Centre, Agriculture and Food Department Authority

Three samples were analyzed Samples A and C were samples of albumin exposed to the extraction process described by Tiygi and Miledi (1991) with methanol and di-ethyl-ether as the organic solvents respectively Sample B was a control albumin sample, not exposed to any extraction process BSA fraction V (Sigma, A4919 lot 110H04635) was used here

Positive identification of C14 0, C16 0, C18 0, C18 1 and C18 2 was made in all samples C10 0 was identified at low levels in samples B and C but a split peak was found at the appropriate retention time for sample A A peak close to the retention time for C8 0 was found in all samples but positive identification was not possible

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Dublin City University
Glasnevin
Dublin 9

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REPORT NO. R 03/94 **DATE** 28 February 1994

REPORT ON ANALYSIS OF PROTEIN SAMPLES FOR FATTY ACID CONTENT

Samples

Three (3) samples of isolated protein were received from Ms Joanne Keenan, Dublin City University on 17 January 1994 The samples were stored refrigerated until analysed

Methods

The samples were extracted with methanol (20 ml methanol per g protein) by shaking for 1 hour The extracts were centrifuged (3500 rpm, 10 min) and the supernatant removed The extraction was repeated and the supernatants combined and evaporated at 45°C under a stream of nitrogen

Methyl esters of the fatty acids in the samples were prepared by saponification with alkali and refluxing with boron trifluoride-methanol The methyl esters of the fatty acids were extracted into heptane, the extract concentrated and the fatty acids determined by gas chromatography with a flame ionization detector (FADM 111)

Results

Laboratory ref	R 03/94/1	R 03/94/2	R 03/94/3
Client ref	A	B	C
Weight of sample (g)	0 4549	0 4572	0 3790
Weight of dried extract (g)	0 0574	0 0059	0 0043
Colour of dried extract	white	white	pink
Fatty Acids (%)			
[< C8 0][1]			3 1
[C8 0][2]	5 0	83 1	81 6
[Cx][3]	2 7		
[Cy][4]	3 6		
C10 0	23 1[6]	0 7	0 6
C14 0	6 1	1 3	0 9
C16 0	23 2	5 8	3 7
[Cz][5]	3 4		0 8
C18 0	10 4	2 4	2 1
C18 1	14 6	4 0	3 6
C18 2	7 9	2 7	3 6

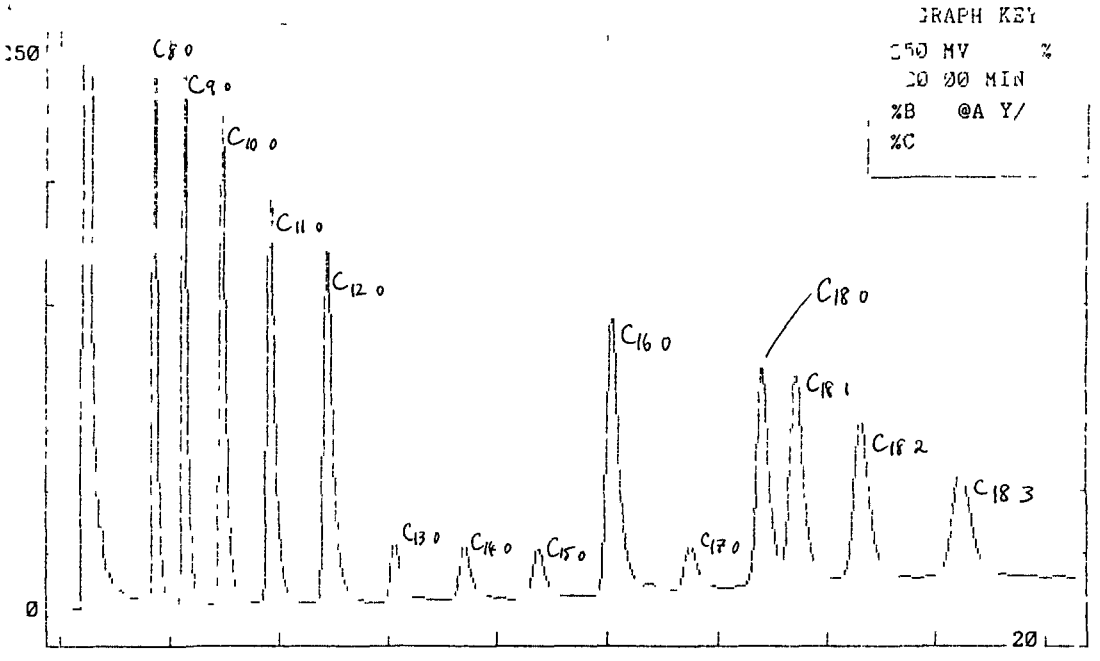
NOTES . [1]Short-chain fatty acid (possibly C6 0)
[2]Tentative identification only as C8 0, retention time slightly different from C8 0 standard
[3 4 5] Peaks not identifiable on basis of retention time of standards
[6]Split peak with average retention time at retention time for C10 0 standard

Michael O'Keeffe

MICHAEL O'KEEFFE
Head, Food Analysis Department

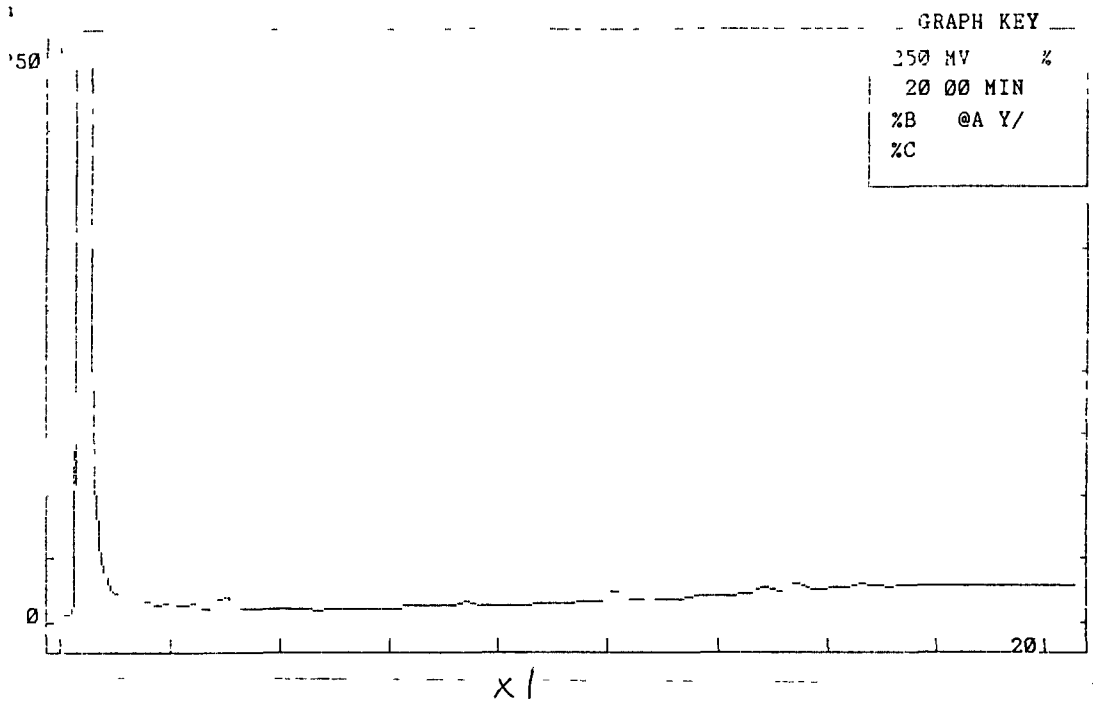
STANDARDS

28/2/94



M. O'Neill

R03/94/1 — A



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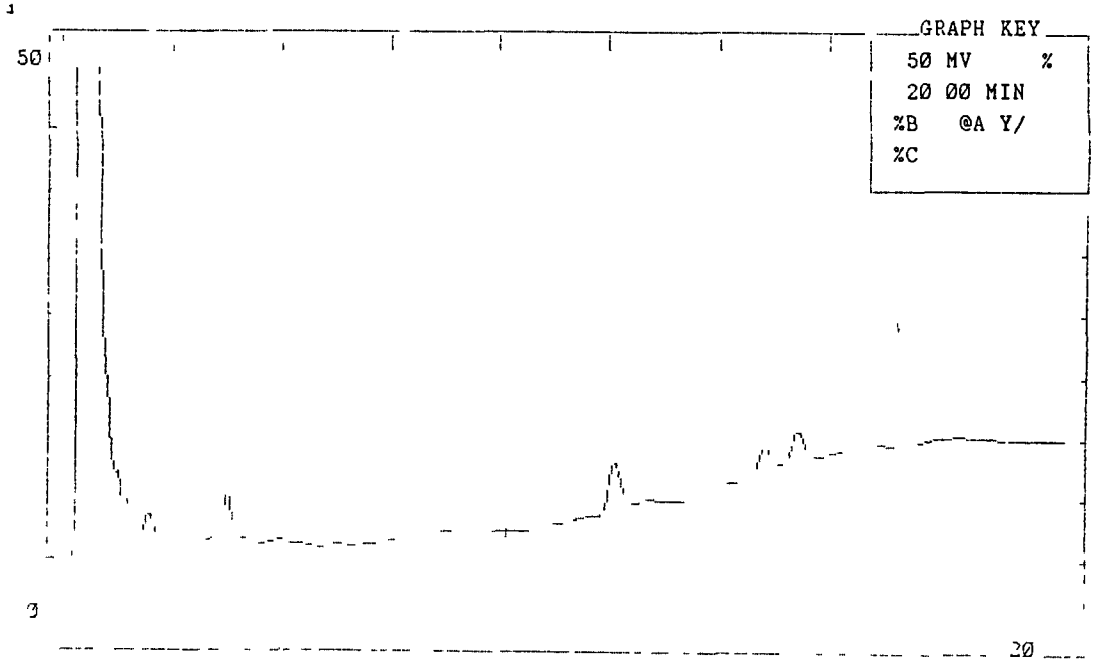

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M Mcelle

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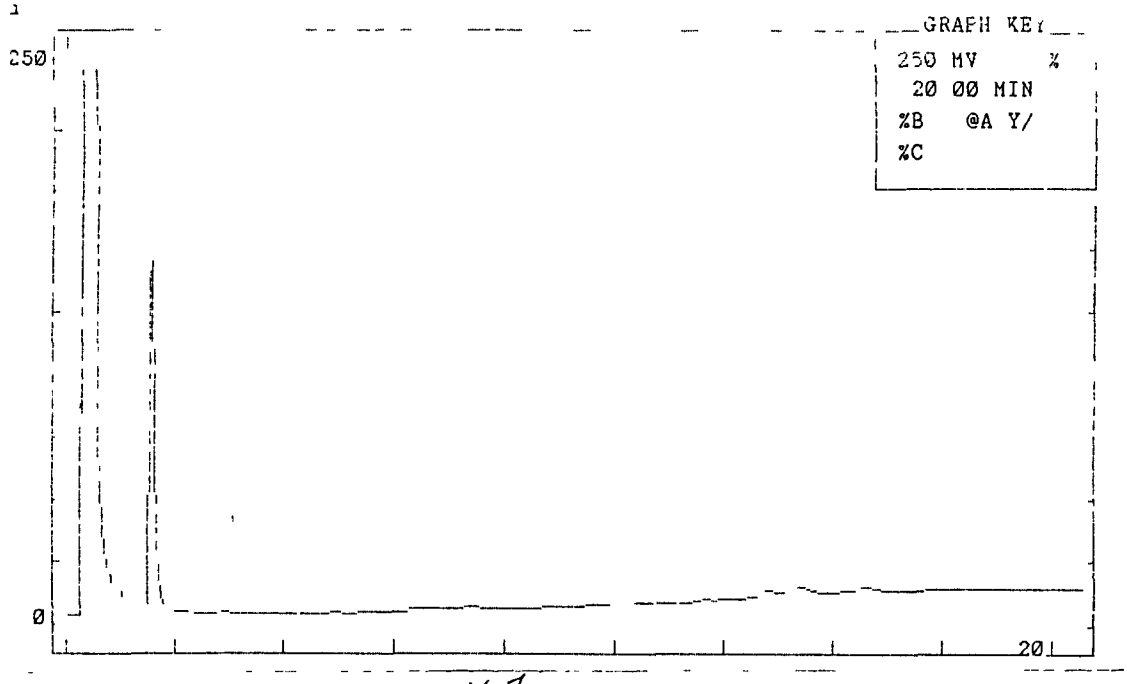

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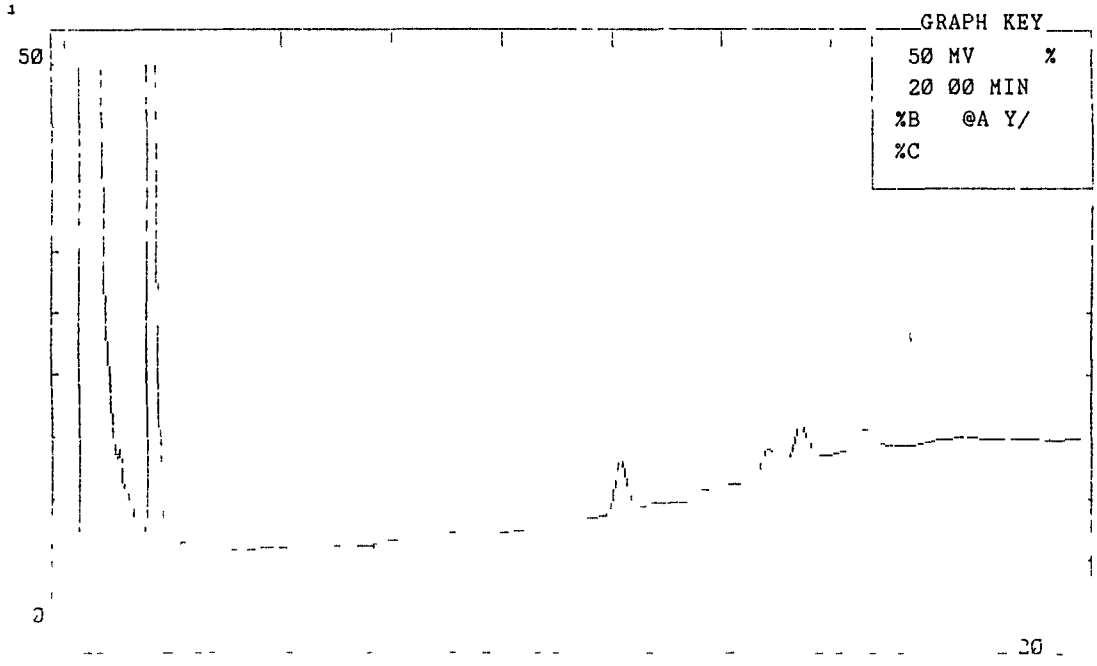
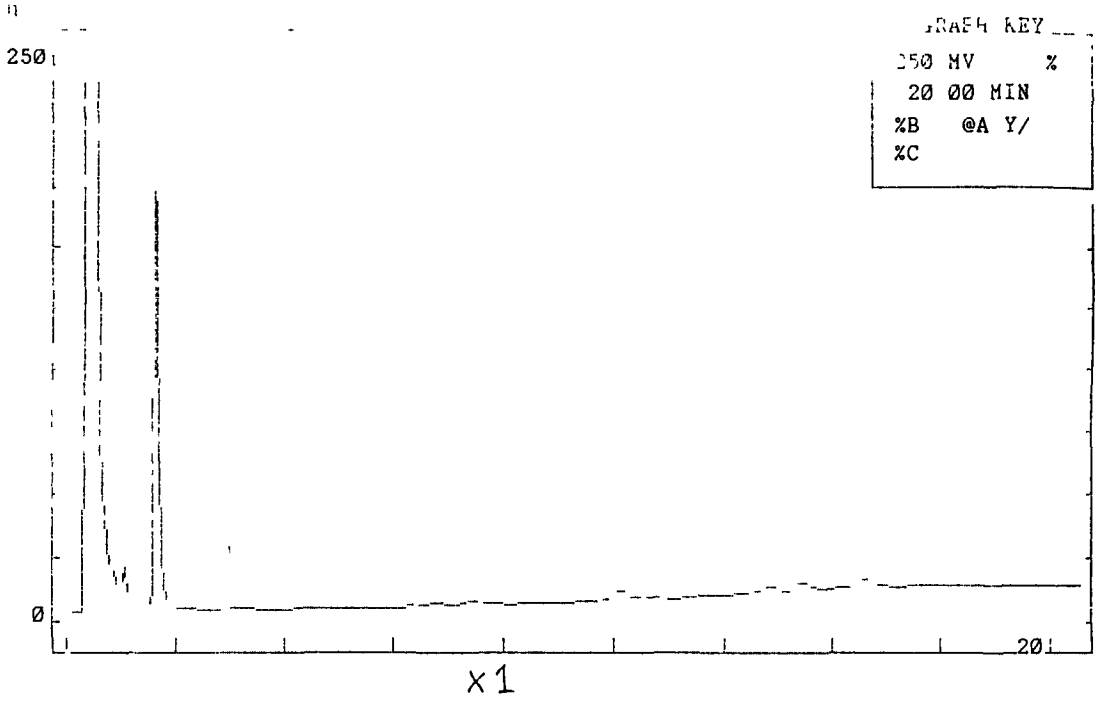


X 5

M. O'Keeffe

R03/94/3 — C



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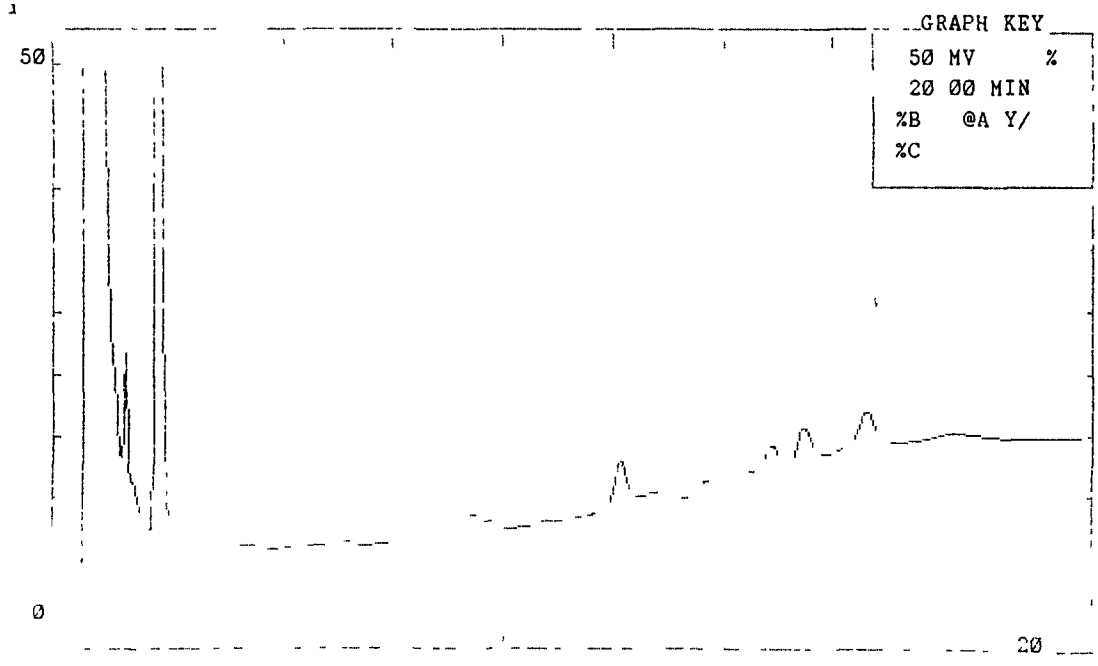
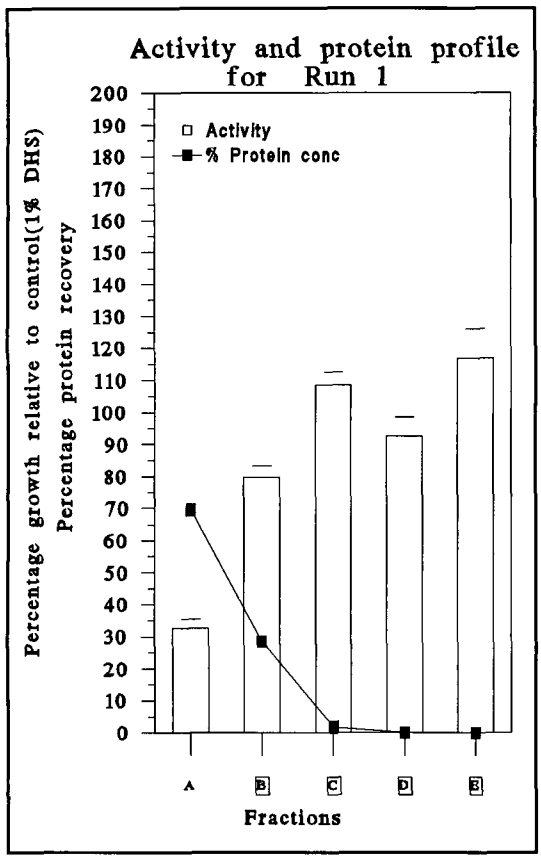


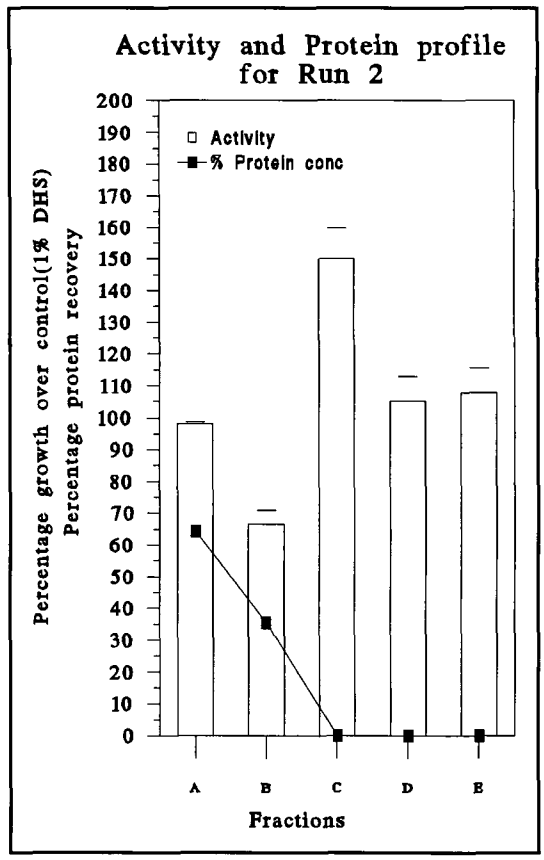
X 5

M. Odeeffe

APPENDIX G

Appendix G shows a comparison of the growth stimulatory activity of heparin sepharose fractions and the protein profiles for Runs 1 to 10

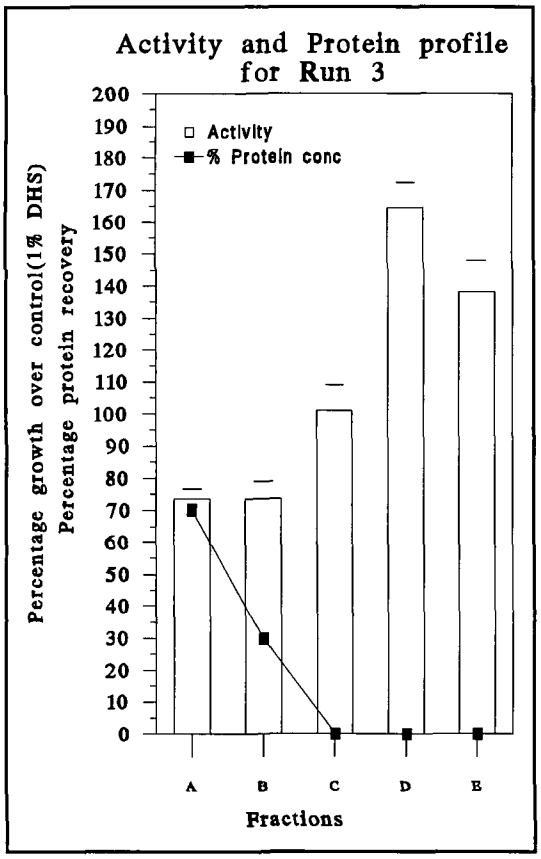


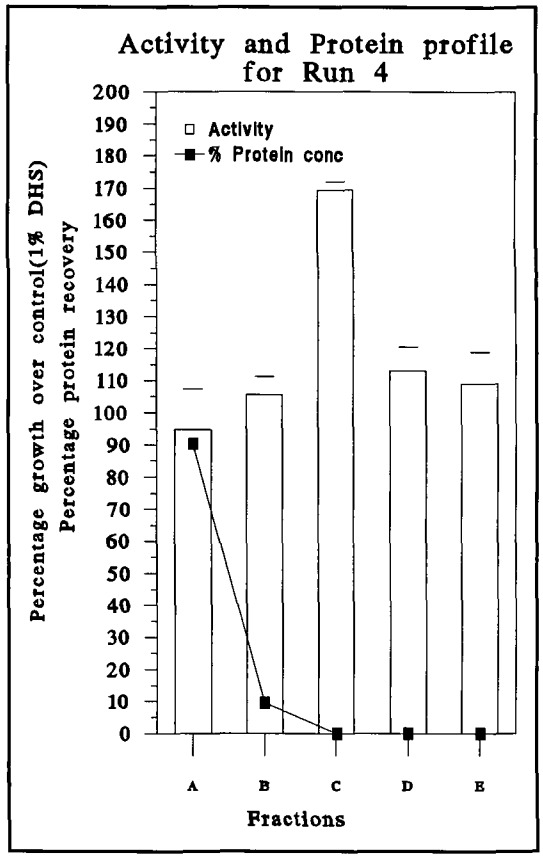


Figures Ga and Gb show the biological activity and the corresponding protein concentration of each fraction for runs 1 and 2 respectively The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were read using Acid phosphatase as the end point The values for the BSA and ATCC controls are shown in Table Ga Results for biological activity are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=8)
Abbreviations A = unbound fraction, B = buffer wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein conc is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column

Table Ga shows the growth response of NRK cells to all the fractions at 5mg/ml albumin(equivalent) concentration

RUN	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
1	100 ± 12 0	134 6 ± 6 82	32 6 ± 2 8	79 5 ± 3 4	109 ± 3 9	92 4 ± 5 9	116 ± 9 2
	100 ± 13 3	134 3 ± 10 4	49 7 ± 5 4	63 8 ± 5 2	97 0 ± 5 3	85 2 ± 3 3	76 2 ± 10 8
	100 ± 8 8	127 7 ± 10 9	48 9 ± 3 9	72 8 ± 4 7	112 ± 8 9	91 7 ± 4 6	84 7 ± 7 5
2	100 ± 8 7	176 2 ± 11 4	98 6 ± 9 4	66 6 ± 4 2	150 ± 9 8	105 ± 7 4	108 ± 7 9
	100 ± 6 8	183 5 ± 12 4	98 0 ± 5 2	76 1 ± 7 9	205 ± 12 4	94 9 ± 5 6	89 2 ± 4 3

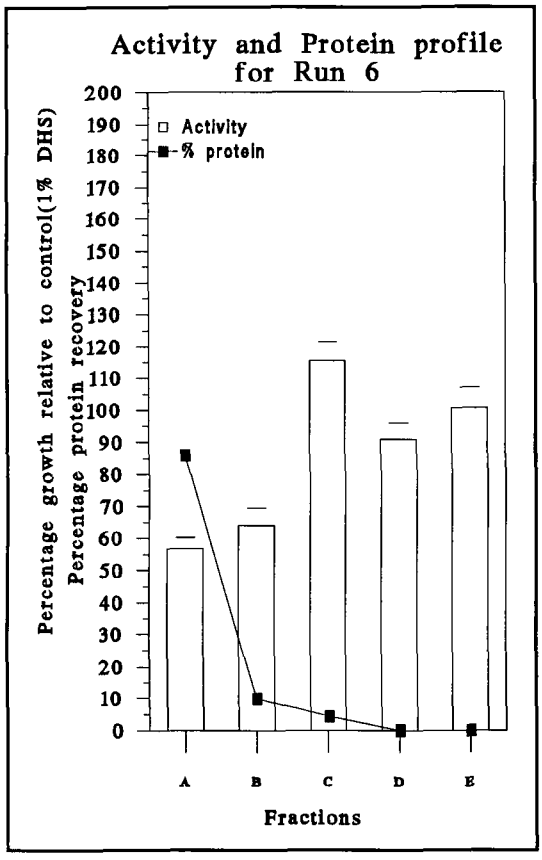


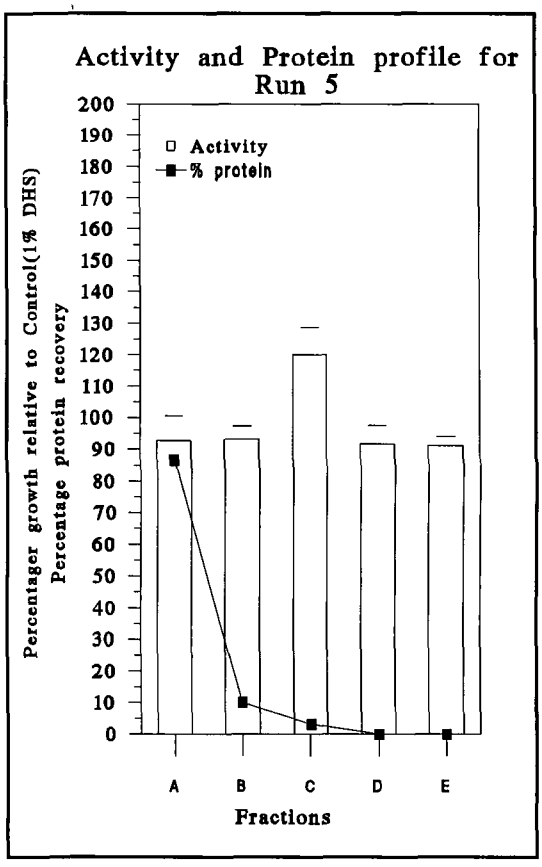


Figures Gc and Gd show the biological activity and the corresponding protein concentration of each fraction for runs 3 and 4 respectively (Assay 1) The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were read using Acid phosphatase as the end point The values for the BSA and ATCC controls are shown in Table Gc Results for biological activity are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations A = unbound fraction, B = buffer wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein conc is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column

Table Gc shows the growth response of NRK cells to all the fractions at 5mg/ml albumin(equivalent) concentration

RUN	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
3	100 ± 3 01	170 3 ± 13 2	73 7 ± 2 9	73 8 ± 3 4	101 ± 7 70	164 ± 7 9	138 ± 9 9
	100 ± 7 46	157 4 ± 11 9	67 3 ± 5 4	72 7 ± 5 2	101 ± 6 53	150 ± 9 3	138 ± 11 8
	100 ± 4 50	138 7 ± 7 82	57 5 ± 3 8	63 1 ± 4 7	105 ± 7 68	141 ± 10	136 ± 11 8
4	100 ± 6 58	148 5 ± 9 70	94 7 ± 12	105 ± 4 2	169 ± 14 4	113 ± 7 3	109 ± 10 2
	100 ± 5 58	129 6 ± 4 43	94 6 ± 6 3	100 ± 7 9	160 ± 10 3	117 ± 5 7	130 ± 3 0
	100 ± 4 46	110 6 ± 5 72	74 4 ± 5 7	125 ± 6 9	161 ± 12 4	119 ± 5 9	126 ± 9 6

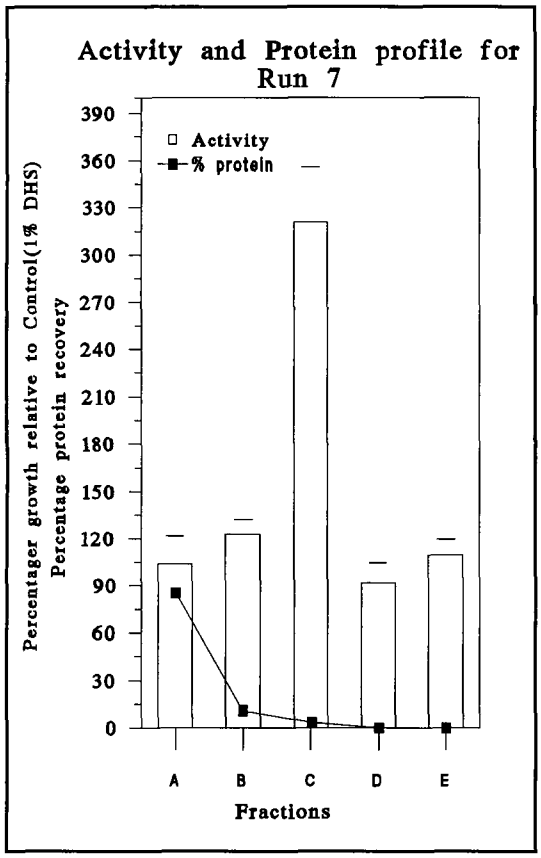


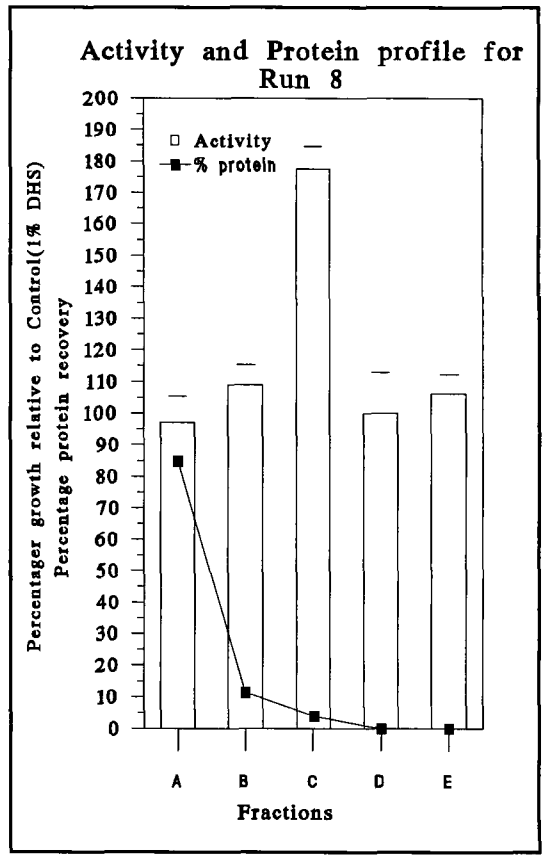


Figures Ge and Gf show the biological activity and the corresponding protein concentration of each fraction for runs 5 and 6 respectively The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were read using Acid phosphatase as the end point The values for the BSA and ATCC controls are shown in Table Ge Results for biological activity are expressed as the percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations A = unbound fraction, B = buffer wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein conc is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column

Table Ge shows the growth response of NRK cells to all the fractions at 5mg/ml albumin(equivalent) concentration

RUN	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
5	100 ± 4 19	133 9 ± 11 2	56 9 ± 3 5	63 9 ± 5 5	115 ± 6 0	90 9 ± 4 9	101 ± 6 20
	100 ± 5 32	127 1 ± 8 39	53 7 ± 1 9	75 6 ± 4 4	121 ± 9 4	99 9 ± 7 0	108 ± 6 64
	100 ± 5 14	129 0 ± 4 74	55 1 ± 3 6	78 7 ± 1 6	122 ± 4 6	93 8 ± 5 9	103 ± 3 96
6	100 ± 3 24	164 8 ± 9 13	92 7 ± 8 0	93 0 ± 4 2	120 ± 8 6	91 7 ± 5 9	91 1 ± 2 9
	100 ± 6 37	144 7 ± 9 09	91 1 ± 5 4	88 1 ± 4 4	94 6 ± 7 0	75 7 ± 4 8	94 1 ± 3 6
	100 ± 5 17	----------	96 8 ± 5 4	84 9 ± 7 2	115 ± 4 12	89 9 ± 3 8	--------

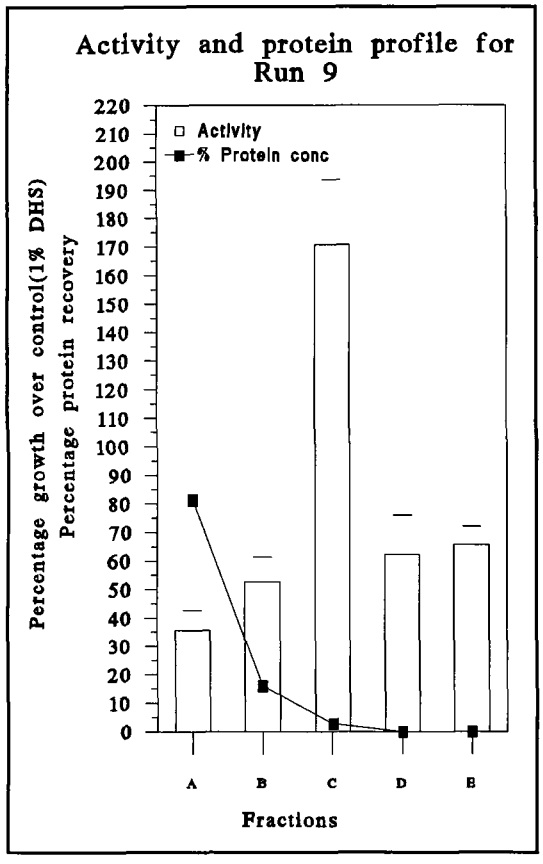


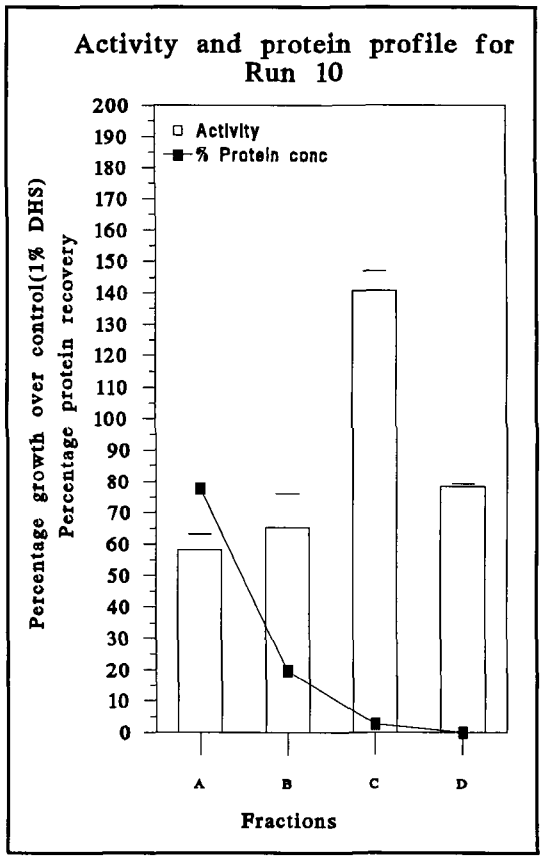


Figures Gg and Gh show the biological activity and the corresponding protein concentration of each fraction for runs 7 and 8 respectively The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were taken down using Dye elution as the end point The values for the BSA and ATCC controls are shown in Table Gg Results for biological activity are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations A = unbound fraction, B = buffer wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein conc is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column

Table Gg shows the growth response of NRK cells to all the fractions at 5mg/ml albumin(equivalent) concentration

RUN	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
7	100 ± 13 6	205 3 ± 11 9	103 ± 18 1	123 ± 9 6	321 ± 35 2	91 9 ± 12	109 ± 9 67
	100 ± 9 67	201 5 ± 28 0	94 9 ± 16	86 2 ± 10	316 ± 24 7	65 8 ± 4 6	97 6 ± 4 35
	100 ± 9 15	273 7 ± 16 9	76 3 ± 6 4	105 ± 11	318 ± 29 9	116 ± 8 07	249 ± 18 1
8	100 ± 13 5	286 9 ± 28 3	97 0 ± 8 4	109 ± 6 4	177 ± 7 21	100 ± 12 8	106 ± 6 12
	100 ± 7 95	201 9 ± 17 0	106 ± 8 6	105 ± 10	170 ± 22 6	137 ± 16 9	121 ± 4 11
	100 ± 10 6	247 8 ± 26 8	102 ± 18	92 9 ± 22	210 ± 23 4	97 4 ± 6 6	98 8 ± 17 3





Figures Gi and Gj show the biological activity and the corresponding protein concentration of each fraction for runs 9 and 10 respectively The biological activity was measured on NRK cells at a concentration of 5mg/ml Assays were taken down using dye elution as the end point The values for the BSA and ATCC controls are shown in Table Gi Results for biological activity are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Abbreviations A = unbound fraction, B = Buffer Wash, C = 0 5M NaCl, D = 1 0M NaCl and E = 2 0M NaCl wash % Protein conc is the protein concentration (determined spectrophotometrically), present in each fraction as a percentage of the total protein recovered from the column Note no value for the 2 0M NaCl wash existed for run 10 as a problem arose with the biopilot and the 2 0M NaCl wash was not initiated

Table Gi shows the growth response of NRK cells to all the fractions at 5mg/ml albumin(equivalent) concentration

RUN	ATCC Control	BSA Control	Unbound Fraction	Buffer Wash	0 5M NaCl Wash	1 0M NaCl Wash	2 0M NaCl Wash
9	100 ± 3 21	175 0 ± 23 7	45 4 ± 18	79 5 ± 9 6	176 ± 11 2	71 2 ± 3 5	92 4 ± 12 5
	100 ± 10 1	196 4 ± 10 3	35 7 ± 7 1	52 7 ± 8 6	171 ± 22 9	62 1 ± 13 8	65 5 ± 6 60
10	100 ± 2 32	349 7 ± 26 5	73 8 ± 11 6	74 3 ± 5 0	337 ± 16 5	85 6 ± 5 39	-------
	100 ± 6 47	188 5 ± 19 5	58 1 ± 5 07	65 1 ± 10	141 ± 6 25	78 2 ± 1 01	-------

APPENDIX H

Appendix H shows the growth response of NRK cells to a dilution curve of each fraction obtained from Run 5 on the HS column (Assay 1) Results are expressed as the average percentage growth relative to control (1% DHS) ± standard deviation (n=8) Acid phosphatase was used as the end point The results for three independent assays are shown in Tables Ha to c Abbreviations A1, A2 and A3 refer to assays 1, 2 and 3 respectively

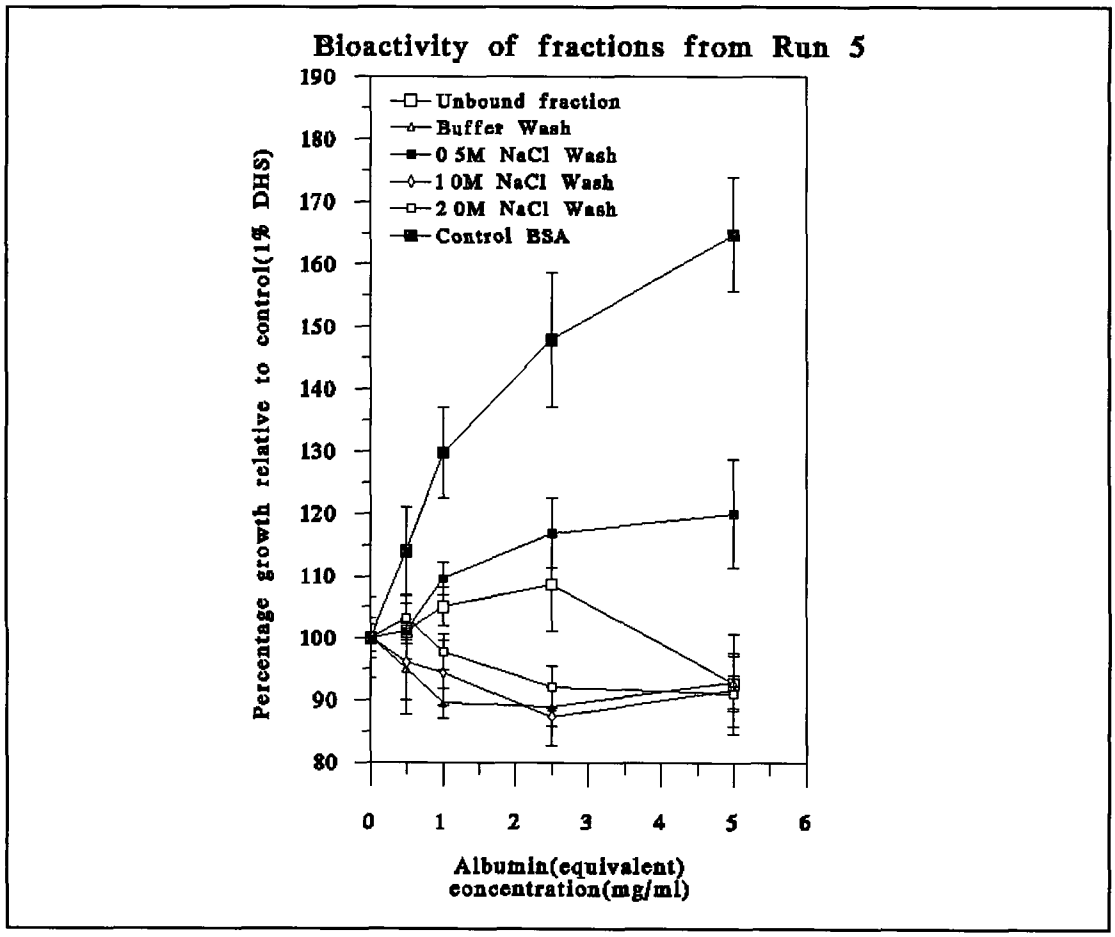


Figure H Growth response of NRK cells in low serum-supplemented medium (1% DHS) to all fractions at albumin (equivalent) concentrations

Table Ha Growth response of NRK cells to all fractions at albumin (equivalent) concentration for assay 1 (mg/ml)

A1	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0	100 ± 2 22	100 0 ± 3 24	100 0 ± 3 24	100 0 ± 6 51	100 0 ± 6 51	100 0 ± 2 22
0 5	114 ± 6 98	101 0 ± 4 54	95 10 ± 7 34	100 7 ± 1 99	96 00 ± 5 96	103 2 ± 3 50
1 0	130 ± 7 30	105 0 ± 3 15	89 51 ± 2 45	109 6 ± 2 65	94 40 ± 5 30	97 77 ± 2 86
2 5	148 ± 10 8	108 7 ± 7 69	88 88 ± 3 15	116 9 ± 5 63	87 40 ± 4 64	92 10 ± 3 35
5 0	165 ± 9 13	92 66 ± 8 04	93 00 ± 4 19	120 0 ± 8 64	91 72 ± 5 96	91 11 ± 2 86

Table Hb Growth response of NRK cells to all fractions at albumin (equivalent) concentration for assay 2 (mg/ml)

A2	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0	100 ± 5 02	100 0 ± 6 06	100 0 ± 6 06	100 0 ± 6 37	100 0 ± 6 37	100 0 ± 5 02
0 5	114 ± 4 74	101 5 ± 4 23	91 50 ± 6 18	91 90 ± 3 82	88 41 ± 4 45	101 0 ± 3 56
1 0	130 ± 5 50	102 7 ± 5 40	85 50 ± 5 40	92 87 ± 5 72	83 96 ± 3 50	95 26 ± 3 56
2 5	136 ± 7 11	100 4 ± 7 33	84 17 ± 8 38	96 68 ± 6 99	76 92 ± 1 94	93 67 ± 4 77
5 0	145 ± 9 09	91 10 ± 5 40	88 09 ± 4 42	94 46 ± 6 99	75 69 ± 4 77	94 07 ± 3 56

Table Hc Growth response of NRK cells to all fractions at albumin (equivalent) concentration for assay 3 (mg/ml)

A3	BSA Control	Unbound fraction	Buffer Wash	0 5M NaCl Wash	1M NaCl Wash	2M NaCl Wash
0 0	----------	100 0 ± 5 17	100 0 ± 5 17	100 0 ± 3 64	100 0 ± 3 64	----------
0 5	----------	102 9 ± 3 58	90 68 ± 4 66	96 01 ± 3 80	91 60 ± 4 13	----------
1 0	----------	102 1 ± 4 30	82 84 ± 2 21	96 97 ± 4 12	89 90 ± 2 85	----------
2 5	----------	105 7 ± 6 09	88 17 ± 3 94	106 4 ± 6 34	84 30 ± 2 85	----------
5 0	----------	96 77 ± 5 38	84 95 ± 7 17	114 7 ± 4 12	89 99 ± 3 80	----------

APPENDIX I

Appendix I shows the growth response of NRK cells to undiafiltered albumin and albumin samples, diafiltered through an R5 or R1 diafilters (Amicon) which have a molecular weight cut off of 5,000 and 1,000 respectively Acid phosphatase was used as the end point As the 1% DHS control was used as the zero blank when reading the acid phosphatase absorbances, results are shown as absorbance readings (dual wavelenght of 405 and 620nm)

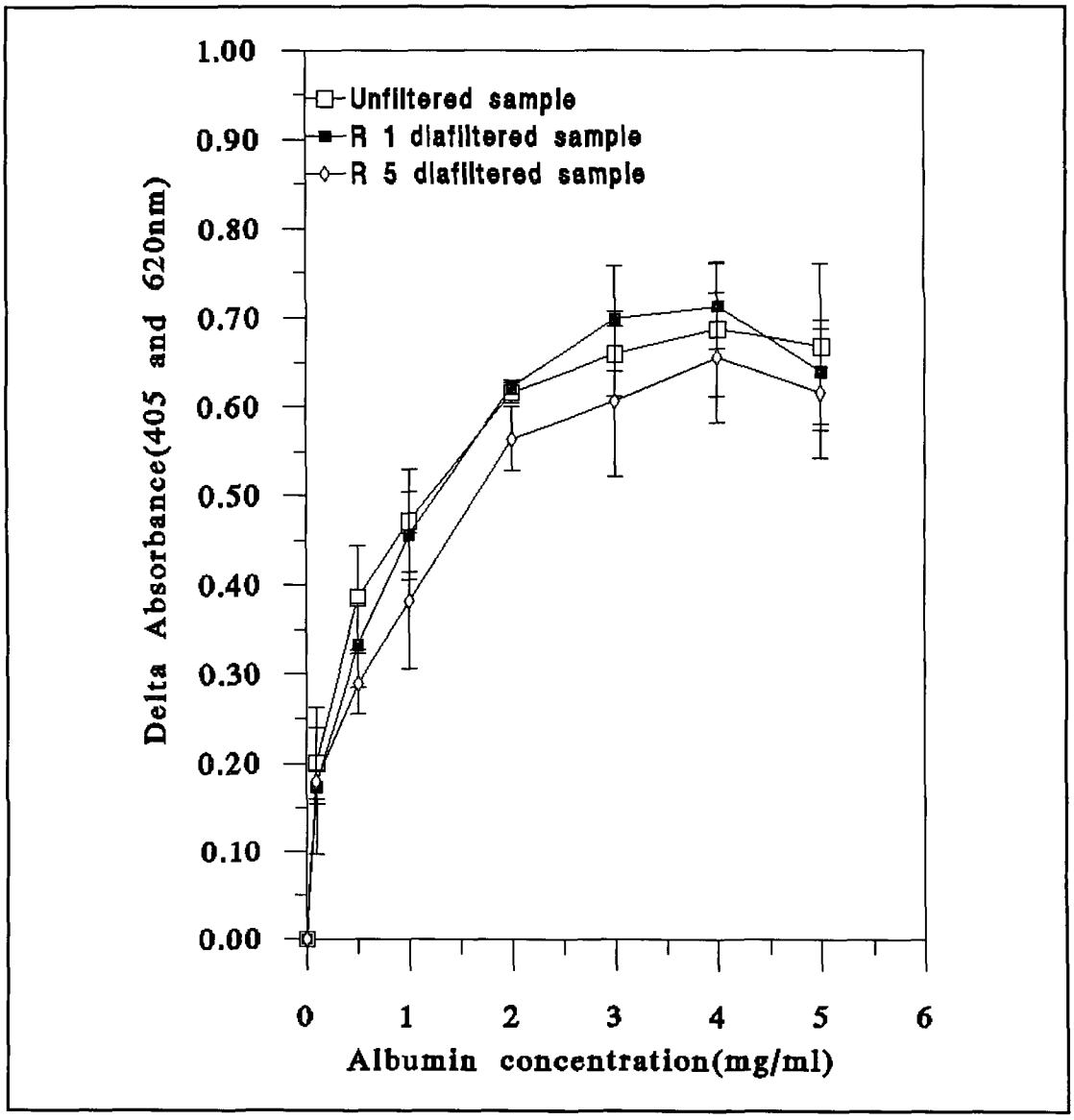


Figure I Effect of Diafiltration on the growth stimulatory activity of BSA fraction V

APPENDIX J

Appendix J lists the trace elements added to the SFM for CHOK1 cells to ensure batch to batch consistency (Mendiaz *et al* , 1986) Elements are at μM final concentration

Element	Conc
$CuSO_4$ $5H_2O$	$1x10^{-3}$
$MnSO_4$ $5H_2O$	$1x10^{-3}$
Na_2SiO_3	$5x10^{-2}$
NH_4VO_3	$5x10^{-3}$
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	$1x10^{3}$
$NiCl_2$ $6H_2O$	$5x10^{-4}$
$SnCl_2$ H_2O	$5x10^{-4}$
$ZnSO_4$ $7H_2O$	$5x10^{-2}$

APPENDIX K

Appendix K shows the typical morphology of CHOK1 cells in serum-free and serum-supplemented medium.

Figure A: Confluent CHOK1 cells in SSM. Magnification 100x.

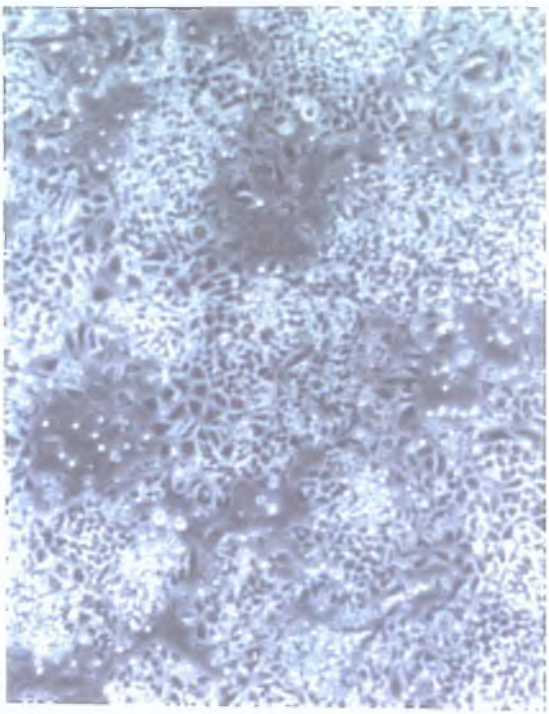


Figure B: CHOK1 cells in SFM (including insulin). Magnification 100x.

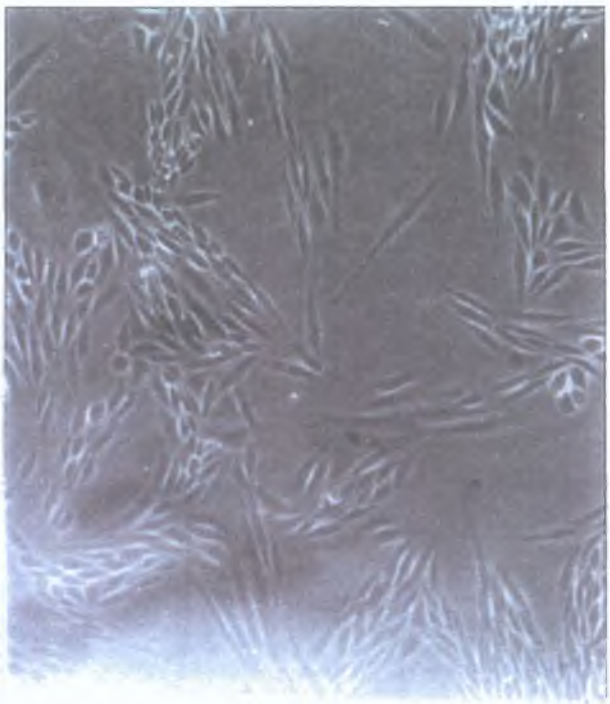


Figure C: CHOK1 cells in SFM without insulin. Magnification 200x.

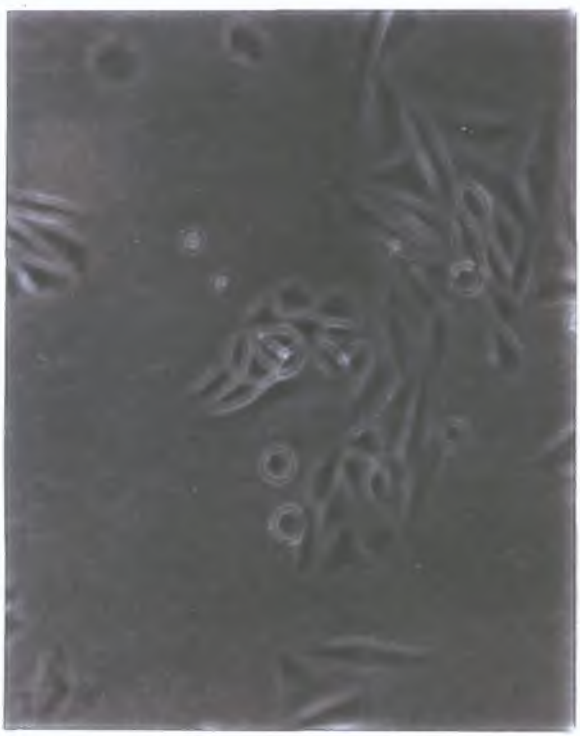


Figure D: CHOK1 cells showing 'ball formation' over extended growth in SFM with insulin Magnification 100x.