Studies on Sterol Carrier Protein

by

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ABSTRACT

Purification of Sterol Carrier Protein (SCP) from rat liver is described. Lipid binding studies indicated that the purified SCP is a fatty acid binding protein. However, no cholesterol binding by SCP was observed. Antibodies were produced in both mice and rabbits to PAG-incorporated SCP and to native SCP and the specificity and sensitivity of two of the antisera was determined to be adequate for development of an ELISA for SCP. Further treatment of the selected antisera is discussed with a view to development of a sandwich ELISA for SCP.

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LITERATURE REVIEW

1. INTRODUCTION

Numerous small hydrophobic molecules are involved in the metabolism of the living cell.. These molecules must often transfer from one cellular compartment to another as they fulfil their biological function. Within the aqueous environment of the cell, the diffusion of hydrophobic molecules would be a slow process. It is not surprising, therefore, that much interest has been generated recently in a family of intracellular low molecular weight soluble proteins which have been implicated in the transport of small hydrophobic molecules within the cell.

The earliest report of such a protein was by Dempsey *et al.*, (1964) who noted that an unknown cytosolic protein was required for the later stages of cholesterol biosynthesis. This protein was subsequently called squalene and sterol carrier protein (SCP) by Ritter and Dempsey (1971). Numerous similar proteins have since been described and are listed, along with their reported amino acid oomposition, in Table 1.1. However, as will be shown later, it now appears that some of the proteins which have been isolated by different groups and given different names are identical proteins. This results in a certain confusion in the literature relating to this area.

To date most information is available on the protein known as SCP. This protein is unique among this group of proteins in that it has also been found in serum and is, therefore, not exclusively intracellular. Its association with the lipoprotein complexes in serum prompted this study which aims to clarify the lipid binding properties of SCP and begin the development of a more sensitive assay system, for SCP, which would aid investigation of its role in the lipoprotein system of serum.

TABLE 1.1 AMINO ACID COMPOSITION OF SELECTED LOW MOLECULAR WEIGHT HYDROPHOBIC BINDING PROTEINS

A.A.	SCP[a]	DEAE PEAK I[a]	SCP_2[b]	FABP[c]	Z-PROTEIN[d]	nsLTP[e]	PROTEIN A[f]	CRBP[g]
Asp	11	14	13	11	11	11	12	17
Thr	12	9	4	12	12	6	10	7
Ser	5	6	7	6	6	11	6	7
Glu	17	14	14	17	17	15	19	19-20*
Pro	2	4	4	2	2	5	2	4
Gly	13	10	14	12	12	18	12	12
Ala	2	10	11	2	2	10	2	6
1/2 Cys	1	1	1	1	1	1	2	3
Val	12	9	6	12	12	6	10	8
Met	7	3	4	6	7	4	6	4
Ileu	9	5	6	9	9	5	7	4
Leu	7	11	12	6	6	10	6	10
Tyr	3	4	0	3	3	1	2	3
Phe	6	5	7	6	6	5	6	5
Lys	17	19	17	17	17	14	18	12-13*
His	2	2	1	2	2	2	2	3
Arg	2	4	0	2	2	2	2	6
Trp	0	1	1	0	0	1	0	2-3*

a Dempsey, et al., 1981

b Noland, et al., 1980

c Gordon, et al., 1983

d Takahashi, et al., 1982a

e Poorthuis, et al., 1981

f Ketterer, et al., 1976

g Ong and Chytil, 1978

* CRABPis identical to CRBP with these exceptions

1.1. Intracellular Low Molecular Weight Hydrophobic Binding Proteins.

1.1.1 Cellular Retinoid Binding Proteins.

Included in this family of proteins are Cellular Retinol Binding Protein (CRBP) and Cellular Retinoic Acid Binding Protein (CRABP). Both can be isolated from rat liver by a laborious purification process. They have estimated molecular weights of 14,600 and 15,460 Daltons, respectively, as determined by gel electrophoresis in SDS for the former and by amino acid sequencing for the latter, (Ong and Chytil, 1978 and Sundelin et al., 1985). CRBP has been reported as having 134 amino acids while CRABP has 136, details of which can be found in Table 1.1 (Sundelin et al., 1985, 1985a). Partial sequence data have indicated a high degree of homology between both proteins (Sundelin et al., 1985a). Both are ubiquitous and can be recovered from all organs supposedly dependent on Vitamin A for proper functioning (Bashor et al., 1973; Ong and Chytil, 1978 and Sundelin et al., 1985, 1985a). CRBP and CRABP function specifically in the binding of retinol and retinoic acid, respectively. However, Sundelin et al., (1985) suggested that the pattern of tissue distribution indicated a function in the intracellular transport, rather than directly participating in the molecular function, of Vitamin A.

1.1.2 Protein A

Protein A has been isolated from rat liver, as a by-product of the preparation of aminoazodye-binding proteins (Ketterer, et al., 1976). It exists in three different forms each being a single polypeptide of approximately 127 amino acids with an estimated molecular weight of 14,000 Daltons (Ketterer, et al., 1976). These proteins vary only with respect to their isoelectric points of 5.0, 5.9 and 7.6. The three forms have been found to have identical tryptic peptide maps and ultra-violet absorption spectra. Differences between the three forms may be related to differences in fatty acyl-CoA derivatives found in association with them, e.g. palmitoyl-CoA

(Ketterer, et al., 1976). Protein A has been detected, immunologically, in rat intestinal mucosa, myocardium, skeletal muscle and adipose tissue. A role in lipid metabolism, as a result of its ligands, e.g. oleate, palmitate, has been postulated. A known carcinogen, a sulphate ester of N-hydroxy-N-methyl-4-aminoazobenzene, is bound by this protein (Ketterer, et al., 1976). It has been suggested that the mechanism of binding of this carcinogen by Protein A could result in the protein promoting its carcinogenic activity or inhibiting it. If Protein A, in vivo, bound the carcinogen non-covalently then it could act as a transport agent for the active carcinogen; conversely if it binds the carcinogen covalently it could inactivate the carcinogen and therefore protect against carcinogenesis (Ketterer, et al., 1976).

1.1.3 Non-specific Lipid-Transfer Protein (ns L-TP).

ns L-TP was originally isolated, by Bloj and Zilversmit (1977), from rat liver. They had problems of inactivation during isolation which can now be avoided by the use of 2-mercaptoethanol (5mM) and glycerol (10% V/V) in working buffers (van Amerongen, et al., 1985). This protein has an estimated molecular weight of 12,400-14,800 Daltons as determined by single band migration on polyacrylamide gels in SDS (Bloj and Zilversmit, 1977 and Poorthuis, et al., 1981). Structural studies indicate that it is single polypeptide of approximately 120 amino acids (TABLE 1.1) with a pI of 8.6. It has also been isolated from numerous other rat tissues and hepatoma cell lines. The concentration of ns L-TP is lowest in testes compared to all other rat tissues tested (van Amerongen, et al., 1985). A ns L-TP concentration almost double that found in rat testes has been observed in rat ovaries (van Noort, et al., 1986). ns L-TP has been found to be specifically localised in the Leydig cells of the testis and its concentration in these cells is dependent on the plasma levels of lutenizing hormone (van Noort et al., 1986). This suggested that ns L-TP may play a role in the regulation of steroidogenesis possibly by regulation of the availability of cholesterol for the P_{450} side-chain cleavage system. A similar

role for this protein in adrenal steroidogenesis has been suggested by van Amerongen et al., (1985) who reported that ns L-TP stimulated the microsomal esterification of cholesterol and its conversion to pregnenolone by adrenal mitochondria. However, to date, no evidence that this protein specifically binds cholesterol has been presented.

1.1.4 Z-Protein

Z-protein has been isolated from rat liver and its purity verified by single band migration on polyacrylamide gels in the presence of SDS (Trulzsch and Arias, 1981). It is a single polypeptide with a molecular weight of 12,500-14,000 Daltons (as determined by polyacrylamide gel electrophoresis in SDS). Three different forms have been isolated (like Protein A) distinguishable by their different isoelectric points (5.2, 6.0 and 7.3; Trulzsch and Arias, 1981 and Takahashi, et al., 1983). Amino acid sequencing revealed that all forms were almost identical (Table 1.1) having 127 amino acids and one mole of associated fatty acids per mole of protein as isolated. It is believed that endogenously associated fatty acids confer on the protein its different isoelectric points (Takahashi, et al., 1983).

Rat liver and growing hepatomas (Grinstead, et al., 1983) are among the only reported sources of Z-protein. Numerous studies have demonstrated its fatty acid binding capabilities. Thus it would seem reasonable to postulate a role, for Z-protein, in fatty acid metabolism. Studies by Mishkin, et al., (1975), indicated that infusion of perfused liver with flavaspidic acid (340 μmol/kg) or bromsulphalien (360 μmol/kg) both of which bind to Z-protein, led to increased uptake of [^{3}H] oleate but that oleate esterification was decreased. They, therefore, concluded that binding of fatty acids by Z-protein was not obligatory for the uptake of fatty acids by the liver but that it might be involved in fatty acid esterification. Others have reported that Z-protein reverses substrate inhibition of acyl-CoA cholesterol acyl transferase at high concentrations of acyl Co-A substrate. Following this Grinstead, et al., (1983) suggested

that by binding acyl Co-As and other enzyme effectors e.g. free haeme, Z-protein might function to modulate the effects of fluctuations in the concentrations of major cellular metabolites.

1.1.5. Fatty Acid Binding Protein

This protein, known as FABP, was initially identified by its association with radioactively labelled fatty acids in selected protein fractions following gel filtration of rat liver (Ockner, et al., 1972 and Mishkin et al., 1972). Co-migration with coloured organic anions has also been used in its purification (Ockner et al., 1982). Homogeneity of purified FABP is confirmed by single band migration on polyacrylamide gels in SDS (Glatz et al., 1985). FABP is a heat stable protein as evidenced by its purification methods (Billheimer et al., 1984). However, most of its activity can be lost by improper storage (Billheimer and Gaylor, 1980). Trulzsch and Arias (1981) noted that the presence of glycerol (20% V/V) in buffers and the use of thiol protecting agents could prevent the loss of functional activity and precipitation from solution during and after purification. Fournier and Rahim (1983) and Fournier et al., (1983) reported that at concentrations in excess of 50 M, purified pig heart FABP self-aggregated into at least four different molecular forms.

FABP is a single polypeptide of approximately 14,000 Daltons, as determined by gel electrophoresis. Multiple forms have been isolated all having similar amino acid composition (Table 1.1), ultra-violet spectra and being immunochemically identical (as determined by immunodiffusion techniques), (Ketterer et al., 1976; Billheimer et al., 1980 and Trulzsch and Arias, 1981). However, they, like Protein A and Z-protein, have different isoelectric points (pI 4.3 - 7.0). Studies have revealed that various forms have different affinities for long chain fatty acids and it is believed that their endogenous ligands ultimately affect their isoelectric points.

FABPs have been isolated from numerous rat tissues, bovine liver

and intestine, rabbit liver, human adipose tissue, pig heart, mouse preputial gland, monkey lung tissue and chick neural retina (Ockner and Manning, 1974; Haunerland, et al., 1984; Matsushita et al., 1977; Haq et al., 1982; Fournier et al., 1983; Moore and Snyder, 1982; Haq et al., 1983 and Lee and Wiggert, 1984). An inter-tissue and inter species difference in the FABPs isolated has been observed by both immunological and electrophoretic methods (Said and Schulz, 1984; Glatz et al., 1985a; Jagschies et al., 1985 and Lowe et al., 1985). FABPs are usually isolated from the cytosolic fractions of test tissues. Interestingly, intestinal FABP was found in significantly greater quantities in the proximal and middle thirds of jejuno-ileum as opposed to distal third or duodenum. In addition, FABP concentration in the villi was almost three times that in the crypts thus suggesting that cellular location is related to function (Ockner and Manning, 1984).

The primary translation product of rat intestinal FABP mRNA has been found to contain 132 amino acids (estimated molecular weight of 15,062 Daltons), (Alpers et al., 1984). No carbohydrate or lipid moieties have been observed (Haunerland et al., 1984). Secondary structure predictions indicated that α-helical structure is limited to the NH_2-terminal end. FABP is generally considered to possess only one fatty acid binding site per protein molecule (Glatz and Veerkamp, 1983). However, Haunerland et al., (1984) have reported the binding of two moles of oleic acid per mole purified bovine liver FABP.

A diurnal variation of FABP levels in rat liver and intestine has been reported (Glatz et al., 1984). However, in the case of the liver this has been disputed by Bass (1985a). Modulation of the rates of fatty acid uptake and utilization, in the intestine, in response to nutritional, hormonal and pharmacological manipulation correlated with changes in cytosolic content of FABP (Ockner et al., 1979; 1980; Rustow et al., 1982; Kawashima et al., 1982 and Brandes and Arad, 1983). These factors are believed to be more important than photoperiod in diurnal variations of FABP levels. In addition Ockner et al., (1979) observed sex related differences in hepatic FABP levels

(44% higher in females). Subsequent studies have indicated that this difference is related to sex hormonal differences (Ockner et al., 1980). Bass et al., (1985) found that the higher FABP concentrations in females was associated with greater quantities of mRNA in the cell. They concluded that sex differences in hepatic fatty acid metabolism was associated with changes in FABP synthesis, mediated pre-translationally.

An exact role for FABP, regardless of origin, remains undefined. However, the bulk of the evidence available would appear to indicate that its role is quite general and relates primarily to fatty acid transport and metabolism. The cellular location of intestinal FABP and its concentration shifts, in response to changes in diet, appears to indicate an important role in fatty acid uptake and transport. FABP preparations have also been shown to modulate the activities of many enzymes for which long chain fatty acids are either substrates or inhibitors (Glatz et al., 1985). Bass et al., (1984) postulated a role for brain FABP. They suggested that soluble FABPs in the brain act as regulators of synaptosomal Na^+-dependent amino acid uptake by sequestering free fatty acids which are inhibitors of this process.

1.1.6 Sterol Carrier Protein$_2$

SCP_2 isolated from rat liver (Srikantaiah et al., 1976) is heat stable up to 55°C. It is a single polypeptide with an estimated molecular weight of 13,500 Daltons (determined by by polyacrylamide gel electrophoresis). Amino acid analysis has revealed that it is composed of 122 amino acids one of which is tryptophan (Table 1.1). Active SCP_2 has been recovered from gels following polyacrylamide gel electrophoresis (Noland et al., 1980). SCP_2 has also been recovered from rat adrenal cortical cells, luteinized rat ovaries and bovine livers (Vahouny et al., 1985; Tanaka et al., 1984 and Scallen et al., 1985). The activation of microsomal conversion of 7-dehydro-cholesterol to cholesterol has been used to assay individual fractions during the purification of hepatic SCP_2.

A role in cholesterol esterification has been reported (Gavey et al., 1981). Lidstrom-Olsson and Wikvall (1986) recently reported that SCP_2 was functional in the activation of microsomal enzymes involved in bile acid synthesis from cholesterol. They concluded that SCP_2 facilitated the introduction of less polar substrates in bile acid biosynthesis to membrane bound enzymes in crude systems in vitro. . However, the broad ligand specificity of SCP_2 appears not to be consistent with a specific regulatory function in bile acid synthesis. Vahouny et al., (1985) reported that SCP_2 facilitated the transfer of cholesterol from outer to inner mitochondrial membrane in the adrenal. However, while this transfer facilitated adrenal steroidogenesis, it was shown that cardiolipin was the more important activator of the cytochrome P_{450} side chain cleavage (P_{450SCC}) system of adrenal steroidogenesis.

1.1.7 Sterol Carrier Protein

1.1.7.1 Discovery

Early studies to elucidate the mechanism of cholesterol biosynthesis, by microsomal enzymes, revealed that an unknown protein factor was necessary for enzymic activity (Dempsey et al., 1964). By 1969, Dempsey had determined that this protein factor was present in the 105,000g supernatant of rat liver homogenate. Later studies revealed that two proteins were required with molecular weights of 30,000 - 60,000 and 14,000 - 16,000 Daltons. At this stage it was postulated that the former was an aggregate of the latter lower molecular weight protein which was called squalene and sterol carrier protein (SCP) by Ritter and Dempsey (1971). This protein is the subject of the present thesis. In 1973 Ritter and Dempsey demonstrated the absolute requirement for SCP in cholesterol biosynthesis by the use of a purified microsomal enzyme system. Subsequent studies revealed that the small 'aggregates' of SCP were, in fact, a 47,000 Dalton single polypeptide called Sterol Carrier $Protein_1$, (SCP_1), (Noland et al., 1980). The protein described by Ferguson

and Bloch (1977) and called Supernatant Protein Factor (SPF) is thought to be identical to SCP_1, both having been reported to function in the two-step conversion of squalene to lanosterol (Caras and Bloch, 1979, and Scallen et al., 1985). Scallen et al., (1985) have suggested that differences in the purification procedures may result in differences in binding specificity observed in vitro.

1.1.7.2 Structure and Occurrence

Molecular weight determinations of SCP carried out following single band migration on polyacrylamide gels in the presence of SDS indicated that the molecular weight is approximately 16,000 Daltons (Dempsey, 1984). Subsequent studies using cloned cDNA sequences have yielded information about the amino acid sequence and consequently the molecular weight has been more accurately determined as being 14,184 Daltons. It has a pI of 7.0 (Takahashi et al., 1982a; Dempsey, 1985). Chemical and cloned cDNA studies, which have been possible because of the abundance of rat liver mRNA, for SCP have shown that SCP is a single polypeptide chain of 127 amino acids whose amino-terminal methionine is acylated (Dempsey, 1985). (Amino acid composition is detailed in Table 1.1). The absence of tryptophan in SCP is of interest because (i) its absence causes the unusual diagnostic ultra-violet absorption spectrum of SCP with an absorption maximum of 278 nm (Dempsey et al., 1981); and (ii) it is also absent from the proteins FABP (Gordon et al., 1983), Protein A (Ketterer et al., 1976) and Z-protein (Takahashi et al., 1982) all of which have fatty acid binding properties. This latter point may be significant in attempting to relate this family of proteins. In this regard Table 1.1 shows up a striking similarity in the amino acid composition of SCP (Dempsey et al., 1981); FABP (Gordon et al., 1983); Protein A (Ketterer et al., 1976) and Z-protein (Takahashi et al., 1982). There is also a striking similarity between the amino acid composition of DEAE Peak I (Dempsey et al., 1981) and SCP_2 (Noland et al., 1980), (ns L-TP shows some resemblence to this group of proteins (Poorthuis et al., 1981) though not as clear cut). CRBP and CRABP seem to belong to a different group of

proteins.

Secondary structure predictions have indicated that a high proportion of SCP is helical in nature (approximately 40% α-helix) (Dempsey, 1985). Other studies revealed that the single free sulphydryl group is partially buried in the native molecule and is not required for functional activity with membrane bound enzymes catalysing cholesterol synthesis (Dempsey et al., 1981). SCP was found to have no associated carbohydrate, phosphate, amino sugar or sterol moieties (Dempsey et al., 1981). However, SCP has approximately one mole of tightly or covalently bound long chain fatty acid (e.g. palmitic and stearic acid) per mole of protein (Dempsey, 1985). Interestingly no carbohydrate or lipid moieties have been reported in structural studies of FABP either (Haunerland et al., 1984).

The presence of SCP in rat liver was first noted by Dempsey et al., (1964). Subsequently it was isolated and purified from most rat tissues. It is especially abundant in tissues which are active in metabolism (as is true for FABP and Z-protein) e.g. intestine, liver, adrenal, ovary and cardiac muscle. It was also found in kidney, lung, brain, spleen, testes, skeletal muscle, adipose tissue and serum. In the latter it is a very minor component comprising only 0.4% and 0.1% of total serum protein during the dark and light period, respectively (Dempsey, 1984). It has been reported that SCP is present at higher levels (30% higher) in female rat tissues as compared to their male counterparts (Ockner et al., 1982; McGuire et al., 1984). Sex related differences in ns L-TP and FABP levels have also been observed (the concentration of ns L-TP and FABP being 50% and 44% higher respectively, in female rat gonads and liver, (van Noort et al., 1986 and Ockner et al., 1979).

SCP has also been obtained from a variety of other mammalian tissues. Among these are human liver, serum, brain, kidney, leukocytes and tumours, bovine liver and adrenal and mouse livers. Other lower forms of life have also been found to contain SCP. These include protozoa, yeasts, fungi and bacteria,

thus confirming the ubiquitous nature of SCP. It has also been isolated from a number of cell culture systems, e.g. human fibroblasts, rat hepatocytes, mouse macrophages, Chinese hamster ovary fibroblasts, 3T3 adipocytes, mouse L_1 cells and many human and mouse malignant cell lines (Dempsey et al., 1982; Alley et al., 1982). Both ns L-TP and Z-protein have been isolated from hepatoma cell culture systems (van Amerongen et al., 1985 and Grinstead et al., 1983).

The structure and amino acid composition of SCP is remarkably conserved throughout nature. Human liver SCP is very similar to that of rat liver (Table 1.2). Similarly the amino acid composition of yeast SCP (177 amino acids) exhibits a strong similarity to liver SCP, with the exception of a higher abundance of glycine and proline. The strong structural similarities exhibited by SCPs of different sources suggest that they may have diverged from a common ancestral gene (Takahashi et al., 1982).

Studies to determine the subcellular distribution of SCP have revealed that 60% is associated with the cytosolic and 30-35% with the mitochondrial fractions of rat liver and adrenal cells (Conneely et al., 1984). No SCP is present in the microsomal fraction (Dempsey et al., 1982). The remainder (approximately 5%) is associated with the nuclear fraction. With respect to cardiac tissues, SCP appears to be evenly distributed between the cytosolic and mitochondrial fractions. Conneely et al., (1986) have reported that nearly all mitochondrial SCP is present in the inner mitochondrial membrane. Very little has been reported about the subcellular distribution of the other binding proteins. It appears that, in general, their cellular and subcellular location is strongly related to their biological functions.

As mentioned earlier, SCP has been found in low concentrations in serum. Most of this SCP was found to be associated with the serum lipoprotein (Dempsey, 1984a). Serum lipoproteins are the vehicles of transport of hydrophobic lipid molecules in the blood. They are complexes of lipid and protein which are divided

TABLE 1.2 Amino Acid Composition of SCP from Different Sources

AMINO ACID	RAT LIVER [a]	HUMAN LIVER [b]	YEAST [c]
Ala	2	4	13
Arg	2	2	5
Asp, Asn	11	12	20
CM-Cys	1	1	1
Glu, Gln	17	22	15
Gly	12	12	20
His	2	2	4
Ileu	9	10	8
Lys	17	16	14
Met	6	3	2
Phe	6	8	10
Pro	2	2	11
Ser	6	7	12
Thr	12	12	11
Trp	0	0	0
Tyr	3	2	5
Val	12	10	16

a From cloned cDNA sequence - Dempsey, 1985.

b By amino acid analysis - Dempsey et al., 1981.

c By amino acid analysis - Dempsey, 1984.

into four main classes on the basis of the proportion of lipid and hence their density;- chylomicrons; very low density lipoproteins (VLDL); low density lipoproteins (LDL) and high density lipoproteins (HDL). The major characteristics of the lipoprotein density classes are shown in Table 1.3.

Studies on the distribution of serum SCP between the lipoprotein density classes have shown that most was associated with the HDL fraction (70%), while only 10% was associated with the LDL fraction. This finding was particularly interesting in the light of the now widely accepted protective role of HDL against atherosclerosis. The mechanism of association between SCP and the serum lipoproteins is as yet undetermined. The present study is aimed at a clarification of the lipid binding properties of SCP. This should shed light on the relationship between SCP and the serum lipoproteins. The importance of understanding this relationship between SCP and serum lipoproteins is confirmed by emerging evidence that serum SCP levels are elevated in individuals suffering from atherosclerosis (unpublished data). Progress in such investigations would be speeded up if a rapid sensitive method for quantitation of SCP was available. This study also aimed to set up an enzyme immunoassay for SCP.

1.1.7.3 Synthesis and Control of SCP Levels

A dramatic biphasic variation of SCP levels has been observed in both liver and adrenals. SCP concentrations range from 2% to 12% of total soluble protein between the mid-light and the mid-dark period, respectively (Song, 1981). It seems, however, that cellular distribution is not affected by diurnal changes (Dempsey *et al.*, 1982). McGuire *et al.*, (1984) noted that a 7-fold increase in SCP (i.e. from 1-7mg/g of liver) occurs in the dark period, peaking at the mid-point and returning to basal levels by the beginning of the light period. A similar but smaller pattern of variation in SCP concentrations has been observed during the light cycle. A similar duirnal variation has been observed in studies of liver and intestinal FABP (Glatz *et al.*, 1984). However, as already mentioned, Bass *et al.*,

TABLE 1.3 MAJOR CLASSES OF HUMAN LIPOPROTEINS

	Chylomicrons	Very Low Density Lipoproteins (VLDL)	Low-Density Lipoproteins (LDL)	High-Density Lipoproteins (HDL)
Density, g ml^{-1}	<0.94	0.94 - 1.006	1.006 - 1.063	1.063 - 1.21
Particle size, nm	75 - 1,000	30 - 50	20 - 22	75 - 10
Protein, % Dry Weight	1 - 2	10	25	45 - 55
Triacylglycerols, % Dry Weight	80 - 95	55 - 65	10	3
Phospholipids, % Dry Weight	3 - 6	15 - 20	22	30
Cholesterol, free, % Dry Weight	1 - 3	10	8	3
Cholesterol, esterified, % Dry Weight	2 - 4	5	37	15

Adapted from Lehninger

(1985a) failed to observe such a variation when studying hepatic levels of FABP.

It was believed that the most likely level of control of SCP concentration was at the point of gene expression. Therefore, it was necessary to determine whether this control was exerted at the point of gene transcription (mRNA synthesis) or translation (manufacture of the polypeptide from mRNA). Studies by Dempsey et al.,(1982) revealed that changes in SCP levels, in liver, during the diurnal cycle were not accompanied by corresponding alterations in the levels of SCP mRNA. They concluded that regulation was occurring at the translational level. This was confirmed in another study by McGuire et al., (1984) who found that alterations in the relative synthetic rate of SCP accounted for the variations in SCP concentration in the liver and that, although large changes occurred in the relative synthetic rate over 24 hours, no significant changes were observed in the level of SCP mRNA. McGuire et al., (1984) suggested that the degradative rate of SCP was constant during the dark/light cycle. Therefore, they concluded that variations in SCP levels were caused by some mechanism controlling the efficiency of translation of SCP mRNA. Possible mechanisms of translational control include selective modification of mRNA to alter its translation; compartmentalization of mRNA or alteration of rates of initiation and elongation in different physiological states (McGuire et al., 1984).

By contrast, Dempsey (1984) reported that, in some rat tissues, e.g. brain and skeletal muscle, although the synthesis of SCP fluctuated during the diurnal cycle the amount of SCP did not change. They concluded that in these tissues variations in the degradative rate must have been occurring.

Studies on the mechanism of synthesis of SCP have revealed that it is produced as a single polypeptide of 127 amino acids. No pro-form of the protein is synthesized (Dempsey, 1985). This was unexpected as, in general, proteins which will be secreted from the cell are manufactured with an additional amino acid

sequence which guides their export from the cell. This signal sequence is then cleaved to yield the active protein. It seems, therefore, that the secretion of SCP into the serum does not follow the regular route. It may be secreted as part of the lipoprotein particle although this also leaves some questions unexplained.

Due to the fact that the relative rate of SCP synthesis paralleled the diurnal pattern it was believed that the photoperiod had a major influence on SCP synthesis. To establish this a number of experiments were designed to determine what other physiological factors (e.g. diet, hormones) influenced SCP levels. These studies revealed that the photoperiod was not related to SCP levels but merely coincidental. Stewart Hargis et al., (1986) demonstrated clearly that both diet and hormones affected SCP levels and not photoperiod. They showed that after a 12-hour fast the diurnal cycle in SCP levels was present but depressed, in comparison to controls. Following a 48-hour fast the diurnal variation was lost but SCP levels remained at a constant high level throughout the 24-hour dark/light period.

As regards hormonal influences, findings indicated that upon glucose administration (1mg/g body weight) endogenous insulin secretion was increased and SCP levels increased two-fold in less than 30-minutes (Glatz et al., 1984). These findings suggest that insulin is a major regulator of SCP synthesis. However, a direct role for glucose cannot be totally discounted. Further proofs that diet affects SCP levels, rather than photoperiod, have been provided by studies in which the pattern of food intake was altered, i.e. food was given 2 or 4 hours into the dark period. A corresponding shift in the peaking of SCP, as compared to control animals was observed (Stewart et al., 1984).

Additional studies have shown that glucocorticoids are required for maximum expression of insulin stimulated SCP synthesis. These findings also supply an explanation for why SCP levels decrease from the mid-point of the dark period while insulin

levels remain high. A simultaneous decrease in glucocorticoid level occurs at this time (Stewart Hargis et al., 1986).

A similar dietary influence on FABP concentrations in selected animal tissues has been observed. Concentrations have been shown to be responsive to changes in the fat content of diet e.g. following a high fat diet the cytosolic concentrations of FABP also rises in chick intestine (Katongle et al., 1980) and in rat liver, intestine, heart and adipose tissue (Rustow et al. 1982; and Haq et al., 1983). Dietary linoleate was found to decrease both the rate of fatty acid synthesis and the content of FABP in rat liver (Herzberg and Rogerson, 1981). In addition, selected drugs have been shown to affect FABP levels. Clofibrate feeding in rats increases the hepatic content of FABP and, also, the uptake of fatty acids (Renaud et al., 1978). Cholestyramine administration also increases the hepatic cytosolic FABP concentration together with the rate of triacylglycerol biosynthesis (Kempen et al., 1983). Thus diet and drug related effects are also observed in FABP concentration. This represents another common feature between SCP and FABP further indicating a strong relationship between these two proteins if they are not, in fact, identical.

1.1.7.4 Functions of SCP

The primary function attributed to SCP, which led to its discovery, is that it acts as an essential component in the microsomal enzymic conversion of lanosterol to cholesterol. It has been postulated that the mechanism of action of SCP, in cholesterol biosynthesis, is by initial formation of a sterol precursor - SCP complex which contains a number of SCP molecules and has particle weight of approximately 2.5×10^5 - 2×10^6 Daltons. This complex binds with a specific microsomal enzyme. The sterol precursor in the complex is converted to its product. The resulting sterol-SCP complex then combines with the next microsomal enzyme in the sequence leading to cholesterol synthesis. This process continues until, finally, a cholesterol-SCP complex results (Dempsey, 1974). This hypothesis is based on the ability of SCP to bind cholesterol

and other sterols which will be discussed later. Scallen et al., (1985) suggest that it is SCP_2 which is important in conversion of lanosterol to cholesterol while Dempsey et al., (1981) argue that SCP_2 is, in fact, the same protein as that which they call DEAE Protein I (Table 1.1) and amino acid sequencing data would seem to support this belief.

Cholesterol is a precursor of the bile acids, the major ones being cholic and chenodeoxy cholic acids. Consequently, studies were performed to determine by what mechanism the water insoluble intermediates of these synthetic reactions were transported along a series of enzymes in the hepatocyte. Some mechanism had to exist to solubilize the sterols and it was, therefore, postulated that some carrier proteins might function in that respect.

Initial studies by Grabowski et al., (1976) revealed that heat stable protein(s) from the 105,000g rat liver supernatant fraction, bound the bile acid precursor 7α-hydroxy-4-cholesten-3-one and that its conversion to 7, 12α - dihydroxy-4-cholesten-3-one by 12α - hydroxylase was increased in the presence of this protein or proteins. They found that SCP bound several water insoluble precursors of bile acids including 7α-hydroxy-4-cholesten-3-one and increased the apparent activity of 12 α hydroxylase. This finding was not restricted to SCP, in fact rat serum albumin was found to have a similar effect. While these findings do not indicate a unique role for SCP in bile acid synthesis they do suggest that a role for SCP in bile acid synthesis could exist. A role in extra-cellular transport of bile acids has been ruled out because SCP has not been found to bind cholic or chenodeoxycholic acids (Grabowski et al., 1976). No role in bile acid synthesis has been reported for the binding proteins most similar to SCP (i.e. FABP, Z-protein and ns L-TP). A role for SCP_2 in bile acid synthesis has been postulated but until SCP_2 can be shown to be pure and distinct from SCP, the value of these suggestions remains doubtful.

SCP is not synthesised in the adrenals (Dempsey et al., 1984)

but it is found in high concentrations there. Therefore, it would seem reasonable to postulate that SCP also plays a role in adrenal metabolism. Ritter and Dempsey (1971) have demonstrated that SCP has an affinity for pregnenolone. On the basis of these binding studies it has been suggested that a cholesterol - SCP complex may participate in the initial stages of steroid hormone biogenesis in adrenals. An adrenal enzyme, which catalyses the conversion of cholesterol to pregnenolone, was found to have enhanced activity in the presence of partially purified liver SCP prepartions (Kan et al., 1972). Similar effects were observed, by the same authors, with a highly purified liver SCP complex. It was also shown that in response to corticotropin (ACTH), (a hormone which stimulates steroidogenesis in adrenal cortical tissue), SCP uptake, from circulating plasma, was enhanced and simultaneously, transport of cholesterol to the inner mitochondrial membrane occurs.

The conversion of cholesterol to pregnenolone is catalysed by the cytochrome P_{450} cholesterol side chain cleavage (CSCC) system located in the inner mitochondrial membrane. Studies indicate that enhanced transport of cholesterol to the cytochrome $P_{450CSCC}$ system accounts, at least in part, for the increase in steroid synthesis in response to ACTH (Dempsey et al., 1980). The role for SCP in adrenal steroidogenesis would thus appear to be related to the transport of cholesterol from the cytosol to the inner mitochondrial membrane where steroid biogenesis occurs.

A role in steriodogenesis for Z-protein, Protein A and FABP has not been postulated. However, Vahouny et al., (1985) did postulate such a role for SCP_2 but suggested that the observed effect of SCP_2 was more likely to be related to activation of the cytochrome $P_{450CSCC}$ system by cardiolipin than to any direct effect by SCP_2. van Noort et al., (1986) did report that the ns L-TP played a role in the regulation of steroidogenesis by regulation of the availability of cholesterol to the cytochrome $P_{450CSCC}$ system - a role which is, in fact, very similar to that postulated for SCP. This is another common factor linking these proteins which may be indicative that they

are, in fact, identical. However, these data may also be interpreted as suggesting a relationship between the proteins identified as SCP_2 and ns L-TP which might find support in similarities of their primary structures.

To carry out many of the functions ascribed to it, SCP must be able to bind cholesterol and several other steroids. Ritter and Dempsey (1973) reported binding of cholesterol, its esters and numerous free fatty acids by partially purified preparations of SCP following a series of gel filtration experiments. However, Scallen et al (1985) were unable to demonstrate any sterol binding by FABP (a protein which, they suggest and has been shown here, may be identical to SCP). Both, Dempsey et al., (1981) and Gordon et al., (1983) have reported that SCP has a high affinity for long chain fatty acids and consider it to function also as an intracellular fatty acid carrier protein. Others have reported that SCP specifically activates membrane bound enzymes catalyzing long chain fatty acid metabolism. Examples of such enzymes include fatty acyl-CoA ligase and acyl-CoA cholesterol acyltransferase (ACAT) (Daum and Dempsey, 1980). In addition, marked correlations have been observed between SCP levels and rates of fatty acid uptake and utilization for triglycerides and phospholipid during several physiological manipulations (Mishkin et al., 1975 and Ockner et al., 1979).

Thus there is much less controversy over the fatty acid binding role of SCP, a feature it has in common with many of the other structurally similar proteins previously mentioned. A role in fatty acid metabolism and transport has also been postulated for FABP, Z-protein and ns L-TP (Glatz and Veerkamp, 1984; Grinstead et al., 1983 and van Amerongan et al., 1984). It is thus necessary to establish whether SCP is strictly a fatty acid binding protein or if it can also bind sterols, the latter being an important part in its postulated role in steroidogenesis. Hence, this study was undertaken in an attempt to clarify previous reported findings.

1.2. Assay Systems.

1.2.1 Assay of SCP Concentration.

In all of the studies to date one of two assay systems has been used to estimate the concentration of SCP present. The first of these is a spectrophotometric method based on the activation of membranes bound Δ^7 - sterol Δ^5 - dehydrogenase by SCP. In this assay the conversion of Δ^7 - cholestenol to $\Delta^{5,7}$ - cholestadienol by Δ^7 - sterol Δ^5 - dehydrogenase, in the presence or absence of SCP (in the microsomal fraction), is determined by scanning the reaction mixture from 250 nm to 310 nm. The amount of $\Delta^{5,7}$ - cholestadienol synthesized is then determined from the $\varepsilon = 11.8\ m^{-1}\ cm^{-1}$ for $\Delta^{5,7}$ - cholestadienol (in cyclohexane) at 281.5 nm. A unit of SCP activity is the increase over the microsomal fraction without SCP of 1 nmol of $\Delta^{5,7}$ - cholestadienol in 45 minutes (Dempsey et al., 1981).

The second method which has been used in most of the investigations into the roles played by SCP, is an immunoprecipitation assay. This is based on the use of a polyclonal antibody to rat SCP, raised in rabbits (McGuire et al., 1984). In this assay the amount of immunoprecipitated protein in various homogenate concentrations is determined and plotted against total protein. The amount of SCP is estimated by comparison of the initial linear slope obtained against a plot for homogenous SCP.

Although these assays have performed well in the past, a more rapid and more sensitive assay would undoubtedly expedite progress in our knowledge of SCP. To this end, this study reports the production of specific polyclonal antisera to SCP and the demonstration of their usefulness in the establishment of an enzyme linked immuno sorbent assay (ELISA) for SCP.

1.2.2 Immunoassays

Immunoassays have been developed as a result of the recognition of the ability of all vertebrates to produce antibodies to any substance (immunogen) which is foreign, or perceived to be foreign, by the individual animal. An antibody having been produced can then be used in a number of different assay formats to determine antigen concentrations in unknown samples. Among the most widely used assays are Immunoprecipitation; Immunoelectrophoresis, Radioimmunoassays (RIA) and Enzyme Immuno Assays (EIA).

Precipitin assays are based on the fact that when antigen and antibody are present at a certain ratio (equivalence point) bridges form between antigen and antibody. This results in a 3-dimensional network which is insoluble and in agarose gels manifests itself as an opaque precipitate. Precipitin assays, on gels, include single immuno-diffusion (Mancini technique) and double immuno-diffusion (Ouchterlony technique). These methods can best be used to give qualitative information in the detection and identification of antibodies. The precipitin test has been used in a quantitative manner by estimating the protein content of the precipitate at optimal proportions. However, there are drawbacks to this method when dealing with samples which contain unknown antigen concentrations as maximum precipitation occurs only at the equivalence point. In addition large quantities of both antibody and antigen are required rendering such assays unsuitable for detection of minute quantities of antigen in unknown samples.

Immunoelectrophoresis is a modification of immuno-precipitation. The reaction of antigen and antibody occurs in a solid phase (agar gel) but is speeded up by the application of an electric field. The antibody is incorporated into the gel while the antigen is applied to a well. Under the influence of the electric field the antigen migrates to the antibody. Again this system is more suited to yielding qualitative rather than quantitative information about the test antibodies.

RIAs were initially developed by Yalow and Berson in 1960. The basis of the technique is the use of a saturable binding agent (antibody) present in restricted quantities to which a radiolabelled antigen can bind. The introduction of unlabelled antigen by comparative inhibition, reduces the amount of labelled antigen which binds to a degree relative to the concentration of unlabelled antigen. This technique has a very high level of sensitivity and reproducibility and has, therefore, gained widespread use. However, there are a number of drawbacks including the high cost of isotopes and their short shelf life; complex equipment is required for the determination of results and, finally, a strong awareness of the dangers associated with widespread use of isotopes and their subsequent disposal. As an alternative, other methods of antigen labelling have been found which include use of enzymes, free radicals, bacterial phages and fluorescent markers. These latter methods have begun to replace the use of RIAs in recent times.

EIAs can be subdivided into homogenous and heterogenous enzyme immunoassays. In the former a hapten is linked to an enzyme in such a manner that the enzyme is altered when the hapten combines with the antibody. This enzyme alteration is manifested spectrophotometrically following addition of a suitable substrate. In the latter system a separation step is performed where the reacted enzyme labelled component is separated, by washing, from the unreacted enzyme labelled material. Heterogenous EIAs have been further refined by linkage of soluble antigen or antibody to a solid phase to facilitate the separation step. This modification is termed an Enzyme Linked Immuno Sorbent Assay (ELISA).

A number of ELISA systems exist including a competitive inhibition assay and a sandwich assay. In the former a limited quantity of the appropriate antibody is initially bound to the solid phase. A limited amount of enzyme-labelled antigen is then added along with the sample or standard unlabelled antigen. As with RIA the amount of unlabelled antigen binding to the antibody reduces, by competitive inhibition, the binding of the

labelled antigen to a degree which is dependent on the concentration of the unlabelled antigen in the sample.

The sandwich assay system is a multi-tier system where either antibody or antigen can be initially bound to the solid phase. If the antigen is bound to the solid phase, it is then reacted with the appropriate antibody which may be labelled itself or may in turn be reacted with an enzyme-labelled second antibody. This assay design is more suited to the estimation of specific antibody concentration than to antigen estimation. Only a proportion of the protein present binds to the solid phase during the coating step and thus the sensitivity of antigen estimation would be low. The double antibody sandwich ELISA is more suitable for measuring antigen concentrations. In this system an antibody is initially adsorbed to the plate. The excess is washed away following a suitable incubation period. The antigen containing solution is then added and, again, following a suitable incubation time, the plate is washed. Next an enzyme labelled specific antibody is added, incubated and the plate subsequently washed. Finally, enzyme substrate is added and the subsequent colour reaction observed spectrophotometrically, is proportional to the antigen concentration in the sample solution. However, this assay format requires two specific antisera for the antigen in question. The same antiserum would be unlikely to work especially with relatively small antigens (as SCP), which might be expected to have fewer antigenic determinants.

1.3 Aims of This Thesis

The aims of this thesis are therefore:

(i) to isolate and purify SCP from rat liver;
(ii) to clarify some of the lipid binding properties of the pure protein and
(iii) to carry out the preliminary steps in the development of a sensitive ELISA assay system for SCP which would permit its rapid measurement in serum and other biological samples.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Chemicals used for routine laboratory procedures were of AnalaR or analytical grade, unless otherwise stated. General laboratory chemicals were obtained from BDH Chemicals Ltd., Poole, England and Riedal de Haen AG, Hannover, Germany. Bradford reagent was from Bio-Rad Laboratories, Germany. Spectrapor membrane tubing was from Spectrum Medical Industries Inc., U.S.A. Ultra-filtration membranes were from the Amicon Corporation, U.S.A. Santillation grade toluene was from Labscan Ltd., Ireland. Scintillation grade 2,5-diphenyloxazole was from Koch Ltd., Suffolk, England. Radiochemicals were from Amersham International plc., England. All antibodies and immunochemical reagents were obtained from Sigma Chemical Company, England. Complete and incomplete Freunds adjuvants were from Gibco Laboratories, U.S.A. All column packing materials were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

2.1.2 Research Animals

2.1.2.1 Source and Strain

Rats used for isolation of protein were female Wistar rats and were supplied by Ballina Laboratories, Ireland. Rabbits used for immunization were female New Zealand Whites and were supplied by Ballina Laboratories or Becton-Dickenson, Dublin, Ireland. Female Balb C mice were also used for immunization studies and were bred in our own animal houses.

2.1.2.2 Animal Maintenance

All animals were maintained under standard conditions of lighting and heating, 12-hours light and 12-hours dark and at 21^{o}C respectively, unless otherwise stated. All received standard animal foods:- rabbit pellet supplied by Paul and Vincent, Ireland in the case of rabbits and Sow Breeder Nuts from Liam Connolly and Sons Ltd., Ireland in the case of the

rats and mice. All animals were routinely sacrificed upon completion of experiments.

2.2 METHODS

2.2.1 Purification of Sterol Carrier Protein

Purification of SCP was performed at 4°C by the method of Dempsey et al., (1981), unless otherwise stated. Deionized distilled water was used in preparation of all buffers used. 0.1% sodium azide was utilized in all solutions as an antibacterial agent, unless otherwise stated. All glassware used during purification was coated in polyethylene glycol 20,000 (Kramer et al., 1976).

2.2.1.1 Protein Extraction

Female Wistar rats, approximately 200-250g in weight, were maintained under conditions of constant light (to increase SCP levels, Dempsey, 1984) for 3- to 4- days prior to being sacrificed by stunning and exsanguination. The livers were excised and stored in ice-cold 0.2M phosphate buffer, pH 7.4, prior to homogenization. These were weighed and placed in fresh phosphate buffer (2ml/g of liver). The liver was homogenized using a 15 ml Dounce Tissue Grinder. The resultant homogenate was centrifuged at 5,000 rpm for 15-minutes in a refridgerated bench centrifuge. The supernatant from this step was centrifuged at 105,000g for 90 minutes in a Beckmann ultra-centrifuge. The supernatant (cytosol) obtained from this step was filtered through glass wool to remove any traces of fat and then stored at -20°C until required.

2.2.1.2 Gel Filtration

10-15 ml of rat liver cytosol was applied to a Sephadex G-75 column which was equilibrated with 30 mM Tris/HCl buffer, pH 9.0. Sample was eluted at a flow rate of 36 ml/hr. 3 ml fractions were collected and protein content monitored by reading the absorbance at 280 nm. Fractions comprising the second protein peak (G-75 Peak II) were pooled and stored at -20°C prior to ion-exchange chromatography.

2.2.1.3 DEAE - Cellulose Chromatography

The DEAE-cellulose column was equilibrated with 30 mM Tris/HCl buffer, pH 9.0, prior to sample application. 150 ml of the second protein peak, obtained from the gel filtration procedure, was applied and eluted at a flow rate of 330 ml/hr. Fractions (3 ml) were routinely monitored for protein content by reading the absorbance at 280 nm. The first peak (DEAE - Peak I) having been eluted, the mobile phase was changed to 30 mM Tris/HCl buffer, pH 9.0, containing 64 mM NaCl. This buffer was used as the mobile phase until the second peak (DEAE-Peak II) was completely eluted. Fractions comprising each peak were pooled and stored, separately, at -20°C prior to protein determination and subsequent preparation for gel electrophoresis.

2.2.1.4 Protein Determination

Protein concentration of all samples was determined using the method of Lowry et al., (1951). A standard curve was prepared using bovine serum albumin (BSA) ranging from 0 - 2.0 mg/ml. Distilled water was used to blank the spectrophotometer prior to reading of all absorbances.

2.2.1.5 Dialysis and Concentration of SCP

To render the protein suitable for gel electrophoresis, binding studies and enzyme immunoassays a dialysis step was required. Dialysis Tubing from Spectraphor, with a molecular weight cut off of 5,000 Daltons was used. This very low molecular weight cut off tubing was used as problems were encountered when using an Amicon ultra-filtration unit where the low molecular weight cut off filters were not found to be effective in concentrating the SCP samples. Routinely 100 ml of DEAE-Peak I and DEAE-Peak II proteins were dialysed against 4 litres of deionized distilled water for 24-hours. Samples for polyacrylamide gel electrophoresis were concentrated by coating the tubing with polyethylene glycol 20,000 and then freeze dried in aliquots. No further concentration step was required for the SCP being used in the binding and enzyme immunoassays.

2.2.1.6 Polyacrylamide Gradient Gel Electrophoresis

Linear gradients of polyacrylamide, 5-35%, were cast in slab moulds. The moulds consisted of glass plates 14 x 8 cm separated by 2.7 mm spacers. The gradient-gel making apparatus and linear gradient maker are depicted in Figure 2.1. Both were supplied by Pharmacia.

2.2.1.6.1 Casting of Gels

Gels were cast by placing 35% (w/v) acrylamide solution, made up in gel buffer - 88.7 mM Tris/81.5 mM boric acid/2.5 M disodium EDTA/0.025% sodium azide, pH 8.3, in Vessel A (Figure 2.1). Gel buffer alone was placed in Vessel B both solutions having previously been degassed. 2.5 ml of initiator (10% (w/v) ammonium persulphate) was added per 100 ml of each solution and 100 µl of 5% (v/v) TEMED was added to each vessel per 100 ml of each solution. Initially an overlay solution, 5% (v/v) methanol, was introduced into the casting chamber. The connection between Vessels A and B was opened and the first portion of the gradient corresponding to 0-5% acrylamide was collected and discarded. The volume discarded was dependent on the number of gels being cast. The remainder of the gradient was then introduced into the casting chamber followed by the underlay solution, 1.0 M sucrose coloured with bromophenol blue. The gels were left to polymerize and then removed and stored under gel buffer at 4°C until required.

2.2.1.6.2 Sample Preparation

Freeze-dried protein samples were reconstituted in sample buffer - 8.87 mM Tris/8.15 mM boric acid/0.25 mM sodium EDTA/5% (v/v) 2-mercaptoethanol/2.0% (w/v) SDS/10% (w/v) sucrose, pH 8.3 (coloured by bromophenol blue to enhance visualization) - to a protein concentration of 3 mg/ml. Samples were solubilized by heating, in sample buffer, to 100°C for 5-minutes.

2.2.1.6.3 Running Conditions

The gels were run in Pharmacia electrophoresis apparatus GE - 2/4, using an LKB 2197 Power Supply. The running buffer used

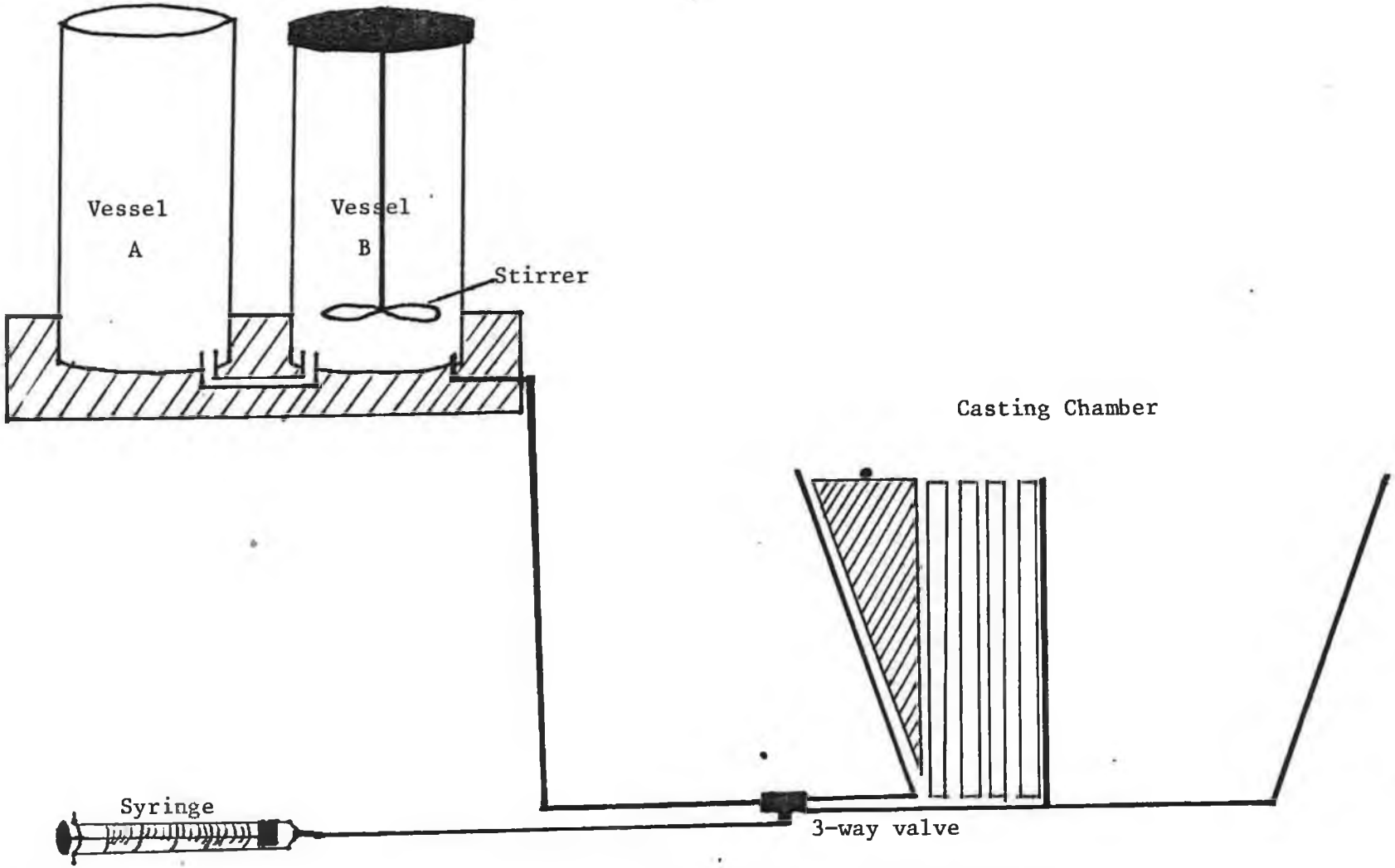

Figure 2.1 Linear gradient maker and gradient gel casting apparatus

was the gel buffer, pH 8.3, with 0.1% SDS. Prior to sample application the gels were pre-run at 150 V for 30-minutes to allow equilibration between the gels and the running buffer. Samples including a set of low molecular weight markers, supplied by Pharmacia, were then applied to the gels, 50µl and 10 µl respectively, and the voltage increased to 300 V until the samples entered the gel (approximately 10 minutes). The voltage was then reduced to 100 V and electrophoresis continued for 12 hours.

2.2.1.6.4 Staining of Gels

The gels were fixed for a minimum of 1 hour in a 15% (w/v) TCA solution. They were stained with a 0.2% (w/v) solution of PAGE blue-83 in water/methanol/acetic acid (6 : 3 : 1, by volume) for 24 hours at room temperature. Gels were then destained in water/methanol/acetic acid (6 : 3 : 1, by volume) until the background was clear. The gels were stored in destaining solution until the SCP bands were removed for immunization.

2.2.1.6.5 Molecular Weight Determinations

The molecular weights of DEAE-Peak I and DEAE-Peak II proteins were determined by comparison of their electrophoretic mobilities with those of the protein standards supplied by Pharmacia, mentioned earlier (2.2.2.6.3.). This was possible because the electrophoretic mobility of a protein subunit on SDS polyacrylamide gradient gels is related to its molecular weight (M.W.) by the following equation.

$$\text{Mobility} \quad \alpha \quad \text{Log}_{10} \ \text{M.W.}$$

2.2.2 Cholesterol and Fatty Acid Binding by SCP

A number of different methods were utilized to establish some of the binding properties of the purified SCP. The chosen ligands for study were cholesterol and oleic acid, both of which were labelled with tritium.

2.2.2.1 Preparation of [^{3}H] - cholesterol solutions

Three different preparations of [^{3}H] - cholesterol were used during these studies.

Firstly a solution with an intended concentration of 300 nmol cholesterol/ml and a specific activity of 5mCi/nmol was used in the Lipidex 1000 studies. The specific activity was obtained by dilution of the labelled cholesterol (9.6Ci/mmol) with unlabelled cholesterol. The appropriate volumes of labelled and unlabelled cholesterol in inorganic solutions were combined, the solvent evaporated under N_2 and the cholesterol redissolved in 10 mls 30mm Tris/HCl buffer (pH 9.0) by sonication for 4 by 1 minute intervals. It soon became apparent that the above solution had a lower concentration than was intended. The reported solubility of cholesterol in aqueous solution at 25^{o}C is 4.7 μM or 4.7 nmol/ml (Haberland and Reynolds 1973). Therefore, this was presumed to be the concentration of the prepared solution. This solution was routinely further diluted 1 in 10 before use giving a solution of 470 pmol/ml.

A solution containing 7.6 pmol cholesterol/ml with a specific activity of 32.9 Ci/mmol was used in the activated charcoal and some gel filtration experiments. The solution was prepared by initial dilution of labelled cholesterol 1 in 20 with toluene. Subsequently, an appropriate volume of the toluene solution was added to a glass vial, dried down and redissolved in 30mM Tris/HCl buffer (pH 9.0). This solution was thoroughly mixed by vortexing immediately prior to use.

In some experiments, which involved the use of gel filtration to separate bound and free cholesterol, a solution of cholestrol in propylene glycol was used. This solution contained 7.6pmol/ml with a specific activity of 32.9 Ci/mmol. This was prepared in a similar manner to the previous solution. All dilute solutions were stored at 4^{o}C for use as required.

2.2.2.2 Preparation of [^{3}H]-oleic acid solution

A solution of 238 pmol of Oleic acid/ml with a specific activity of 4.1 Ci/mmol was prepared as follows. A 1 in 20 dilution of

the labelled oleic acid was initially made with toluene. An appropraite volume of this solution was dried down in a glass vial and redissolved in 1000 times the volume of 30 mM Tris/HCl buffer (pH9.0). The solution was mixed thoroughly by vortexing prior to use. This solution was used in all fatty acid binding studies.

2.2.2.3 Delipidation of SCP

For some experiments the SCP was delipidated prior to use by the method of Chen (1966). 0.005g of activated charcoal was added to 100 mls of SCP (0.1 mg/ml) and the pH lowered to 3.0 by the dropwise addition of 0.2 M HCl. This solution was then placed in an ice-bath and mixed, using a magnetic stirrer for one hour. The charcoal was removed by centrifugation at 20,200 g for 20 minutes in a Sorvall centrifuge with an S-34 rotor at 2°C. The supernatant was collected and the pH brought to 7.0 by the dropwise addition of 0.2 M NaOH. The delipidated solution was stored at 4°C until required and subsequently concentrated, by reverse dialysis against polyethylene glycol 20,000 to a desired concentration of 0.25 mg/ml.

2.2.2.4 Binding Studies Using a Lipidex 1000 Column to Separate Bound and Free Ligand.

To prepare the Lipidex 1000 column, 0.5 g of the powder was allowed to swell overnight in 10 ml of methanol at 4°C. The solution was then degassed and a 1 ml column poured in a 2 ml syringe. This column was stored at 4°C for use as required. Prior to use the column was equilibrated with 10 column volumes of 30 mM Tris/HCl buffer (pH 9.0) at the required running temperature. The flow rate was maintained at 12 ml/hr.

Initially, the ability of the column to bind free cholesterol at 4°C and 30°C, was tested. 300 µl of a cholesterol solution (with a concentration of 470 pmol/ml) in 30 mM Tris/HCl buffer (pH 9.0) was applied to the column and 0.5 ml fractions collected. The unbound cholesterol was eluted with buffer: (30 mM Tris/HCl (pH 9.0)). Change of the mobile phase to methanol resulted in elution of the bound labelled material. 0.3 ml of each fraction was counted for radioactivity in a Beckmann

Scintillation Counter. The scintillation cocktail used was 0.5% (w/v) 2,5-diphenyloxazole in a 2:1 toluene/Triton X-100 solution, 6 ml of which was used per sample. The percent label bound by the column was estimated by calculation of the percentage of total counts in the eluent comprising the second peak.

Protein binding activity was investigated by incubation of the test proteins (400μg BSA and 8.6μg SCP) and labelled cholesterol (94 pmol and 47 pmol, respectively) at 37°C for 30 minutes in polypropylene vials. The mixture was then applied to the Lipidex 1000 column and run as already described at temperatures of 4°C and 30°C. Fractions were monitored for radioactivity as described previously.

2.2.2.5 Binding Studies Using Activated Charcoal to Separate Bound and Free Ligand

The following charcoal suspensions were used during these studies:(A) 0.1% - 1% (w/v) activated (Norit A) charcoal in 0.1M PBS (pH 7.4); (B) Dextran coated charcoal (2.0% (w/v) charcoal, 0.2% (w/v) Dextran T-70) in 0.1M PBS buffer (pH 7.4) with 0.1% (w/v) gelatin added as required.

A similar assay procedure was followed when the binding of both cholesterol and oleic acid by SCP and BSA was being measured using activated charcoal to separate bound and free ligand. The assay was performed in 1.5 ml polypropylene tubes. 200μl of the protein solutions containing 0.1μg to 100μg of SCP or 10μg of BSA were incubated with 100 μl of the labelled cholesterol solution in buffer at a concentration of 7.6 pmol/ml for 30 minutes at 37°C. The final cholesterol concentration was thus 2.5 nM. The vials were then placed in a methanol bath at 0°C and 0.1 ml of one of the ice-cold activated charcoal suspensions, described above, was added to separate bound and free label. The samples were vortexed and incubated at 0°C for 10 minutes and then centrifuged in an Eppendorf microfuge for 2-minutes at 10,000 g. 0.3 ml of the supernatant was counted for radioactivity as described previously. For the estimation of non-specific binding the protein solution was replaced by buffer and a similar protocol followed.

In the oleic acid binding studies 10μg of protein either BSA or SCP in a volume of 200 μl was incubated with 100 μl of the labelled oleic acid solution described above. The final oleic acid concentration was, therefore, 79 nM.

2.2.2.6 Investigation of Gel Filtration as a means to separate Bound and Free Cholesterol

A number of gel filtration columns (1.4 x 20 cm) were prepared using Sephadex G-10, G-25 and G-75 by pre-swelling the respective beads in 30 mM Tris/HCl buffer (pH 9.0), (with 0.005% Sodium Azide as a preservative). The solutions were degassed and the columns packed by elution of several column volumes of buffer.

All columns were stored at room temperature and used as required. The void volume was determined by application of 0.5 ml of a 0.5% Blue Dextran solution to each of the columns. When running the columns 0.5 ml of the second and third cholesterol solutions mentioned earlier was added and eluted at a flow rate of 15 ml/hr. 0.5 ml fractions were collected and assayed for radioactivity as described previously.

2.2.3 Antibody Production and Characterization

Antibodies to SCP, purified in Section 1 (2.2.1) were raised in both mice and rabbits (Ascites production and intradermal injection, respectively). In both cases native SCP and SCP from polyacrylamide gels were used.

2.2.3.1 Production of Antibodies in Ascites Fluid in Mice

Antibody production was induced by intraperitoneal injection of SCP (in either the native form or in polyacrylamide gel slice) into two groups, each comprised of 4-mice, according to the procedure of Tung (1983).

Ten to twelve week old female Balb C mice were used. This age group was chosen because it is reported that in younger mice (six to eight weeks) only small volumes of ascites are produced and in older animals the production of ascites is reported to be

much slower. Females were used as males of this strain tend to be more aggressive.

Animals were immunized with an emulsion of complete Freunds adjuvant and antigen in the ratio of 9 : 1. A stock solution of 5 mg/ml native SCP was prepared following dialysis and concentration as described (2.2.1.5). Bands from polyacrylamide gradient gel electrophoresis gels corresponding to SCP were excised and homogenized in distilled water to a final concentration of 5 mg/ml PAG-incorporated SCP. Immediately prior to injection the emulsion was prepared, by vortexing, using 0.2 ml of the appropriate stock solution and 1.8 ml Freunds complete adjuvant.

0.2 ml of the emulsified immunogen was injected intraperitoneally using a 21-guage, 1.5 inch needle with a 1-ml syringe, into each mouse. This was day 0 of the immunization schedule. This step was repeated on days 14, 21, 28 and 35. In the interim, where ascites fluid development was visually apparent (by swelling of the abdomen) the fluid was collected by the insertion of a sterile 18-guage, 1.5 inch hypodermic needle close to the surface of the peritoneal cavity. On day 42 each mouse was injected intraperitoneally with 0.5 ml pristane. This was the only occasion that pristane was used.

Following collection of the fluid the ascites was filtered through nylon mesh, centrifuged at 2,500 rpm for 15 minutes at 4° C to remove cell debris. The fatty layer was removed using cotton tipped applicators. The ascites was recentrifuged at 15,000 rpm for 20 minutes at 4°C and the supernatant was filtered through glass wool to remove any remaining fat. The ascites was then stored at -20°C until all ascites production was completed.

2.2.3.2 Antibody Production in Rabbits

Antibody production was induced either by intradermal injection of SCP (in either native or PAG-incorporated form) or by sub-cutaneous injection using a 1 : 1 ratio of Freunds complete adjuvant to immunogen. Both methods have been reported to give a good immune response (Hurn and Chantler, 1978). 3-month-old

New Zealand Whites were subjected to the immunization schedule as outlined in Table 2.1. The animals were bled at regular intervals and the serum titre for anti-SCP activity determined using a sandwich ELISA with commercial conjugates. Large bleeds were taken when appropriate, approximately 7 - 10 days after boosting.

Blood was collected from the ear vein in clean dry bottles and allowed to clot at room temperature. Samples were then centrifuged and the serum separated and stored at -20°C for further processing.

2.2.3.3. Enrichment for Immunoglobulin

Enrichment for immunoglobulin was performed by the method of Tung (1983) on the large bleed samples and pooled ascites from each group. Each sample was placed in a beaker on ice and 5N ice cold HCl was added to adjust the pH to 4.5 - 5.1 with continuous stirring to precipitate the fibrinogen. This pH was maintained for 1 hour and the samples were then centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatants were collected and 5 N NaOH was added, dropwise with continuous stirring, to adjust the pH to 7.0 - 7.2. To enrich for immunoglobulin 25% sodium sulphate was added dropwise to a final concentration of 18%. The volume of 25% sodium sulphate was calculated by the following formula:

$$18 = \frac{25x}{(x+y)}$$

Where x = volume of 25% sodium sulphate and y = initial volume of sample. All steps from this stage were performed at room temperature. The samples were allowed to stand for 1 hour prior to centrifugation at 15,000 rpm for 20 minutes at 20°C. The resultant supernatant was discarded and the precipitates were washed once with 13% sodium sulphate and the centrifugation step repeated. The resulting pellets were suspended in a minimal volume of distilled water. The samples were then dialysed against 0.1 M Tris/HCl buffer, pH 8.0, overnight. The antibodies were stored at 4°C for use as required.

TABLE 2.1 Immunization Schedule of Test Rabbits

Animal	Initial Immunization			Days Post Initial Immunization	1st Boost			Days Post Initial Immunization	2nd Boost		
	SCP		Route[a]		SCP		Route		SCP		Route[a]
	Native µg	PAG-Incorp. µg			Native µg	PAG-Incorp. µg			Native µg	PAG-Incorp. µg	
R_{262}		220	I.D.	25		150	S.C.	139		150	S.C.
R_{263}[b]		300	I.D.	25		150	S.C.	-	-		-
R_{279}		150	I.D.	91		75	S.C.	-	-		-
R_{280}		150	I.D.	91		75	S.C.	-	-		-
R_{282}	500		S.C.	68	250		S.C.	-	-		-
R_{283}[b]	360		S.C.	-	-		-	-	-		-
R_{284}	500		I.D.	68	250		S.C.	-	-		-
R_{289}	360		I.D.	68	250		S.C.	-	-		-

a) I.D. = intradermal injection along multiple sites (~20) on the back
S.C. = subcutaneous injection using multiple sites (~4) on the back

b) = animals died of natural causes post immunization

2.2.3.4 Antibody Titre Determination

The antibody titres of each bleed and of enriched immunoglobulin samples were determined. Nunc 96-well, non-sterile polystyrene microtitre plates were coated with 10µg of SCP per well in 200µl of 0.05 M Carbonate buffer, pH 9.5. The plates were incubated at 37°C for 1.5 hours and then washed five times with 0.1 M PBS/0.05% (v/v) TWEEN wash solution. The plates were dried by tapping of plates on tissue paper until all traces of liquid were removed and then 100 µl of the test sample, at increasing dilutions, was added. The plate was incubated at 37°C for 1.5 hours and washed as before. A suitable dilution of a horse-radish peroxidase conjugated anti-rabbit or anti-mouse IgG (as required) was prepared in 0.1 M PBS/0.05% (v/v) TWEEN and 150 µl added per well. The plate was then incubated at 37°C for 2 hours and washed again. The colour was developed by adding 100 µl of substrate solution (1.5 mg O-phenylenediamine (OPD)/0.5 M phosphate/0.1 M citrate buffer, pH 5.0) containing 0.6 µl H_2O_2 per ml) to each well and the plates left in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 25 µl of 20% H_2SO_4 to each well and the $O.D._{490\ nm}$ of each well was read, immediately using an ELISA EL 307 plate reader. The titre was taken as the dilution which gave an $O.D._{490\ nm}$ reading of 1.0 under the standard assay conditions.

2.2.3.5 Determination of the Specificity and Sensitivity of the Antisera

The specificity of selected antisera was determined by coating wells of the microtitre plates with 100 or 1000 µg of selected proteins in a total volume of 200 µl as for coating of SCP. DEAE Peak I protein used was purified by a second passage through the ion-exchange column. The assay was then completed as described in section 2.2.3.4.

The sensitivity of selected antisera was determined by coating wells of the microtitre plate with 0.02-2 µg SCP in a total volume of 200 µl. The assay was then completed as described in section 2.2.3.4.

3 RESULTS.

3.1 Purification of SCP

The purification of SCP was carried out by the method of Dempsey et al., (1981) as outlined in section 2.2.1. Liver cytosol was initially subjected to gel filtration on a Sephadex G-75 column, which has a fractionation range of 3,000 to 70,000 molecular weight. This resulted in separation of the low molecular weight proteins in the cytosol. A typical elution profile is outlined in Figure 3.1.1. The lower molecular weight proteins were present in the second peak called G-75-Peak II protein. Further purification of SCP was achieved by DEAE-ion-exchange chromatography of the pooled fraction comprising G-75-Peak II proteins. SCP, having a pI of 7.1, bound to the anion exchanger - DEAE cellulose - at the running pH of 9.0 and was displaced by increasing the salt concentration. The elution profile resulting from this ion-exchange step is shown in Figure 3.1.2. Generally the bound protein was eluted as a single peak as described by Dempsey et al., (1981). However, where older animals (greater than 6-months) were used as the source of liver cytosol a second small peak, called DEAE-Peak IIa was obtained shortly after change of the mobile phase (Figure 3.1.3). No such peak has been reported by Dempsey et al., (1981). The major peak called DEAE-Peak II consistently corresponded to the position of the SCP peak of Dempsey et al., (1981).

The recovery of protein at each stage was determined as described in section 2.2.1.4 and the results outlined in Table 3.1.1. As expected most of the protein was lost in the gel filtration step. Greater than 80% of the protein applied to the ion-exchange column was routinely recovered with 21% appearing in DEAE-Peak I and 62% in DEAE-Peak II. Assuming that the protein in DEAE-Peak II is pure SCP, this means that 2.3% of the initial protein was recovered as SCP. This corresponds well with the reported values of SCP being 2-8% of total cytosolic protein (Dempsey et al., 1981; Conneely et al., 1984 and Dempsey, 1985).

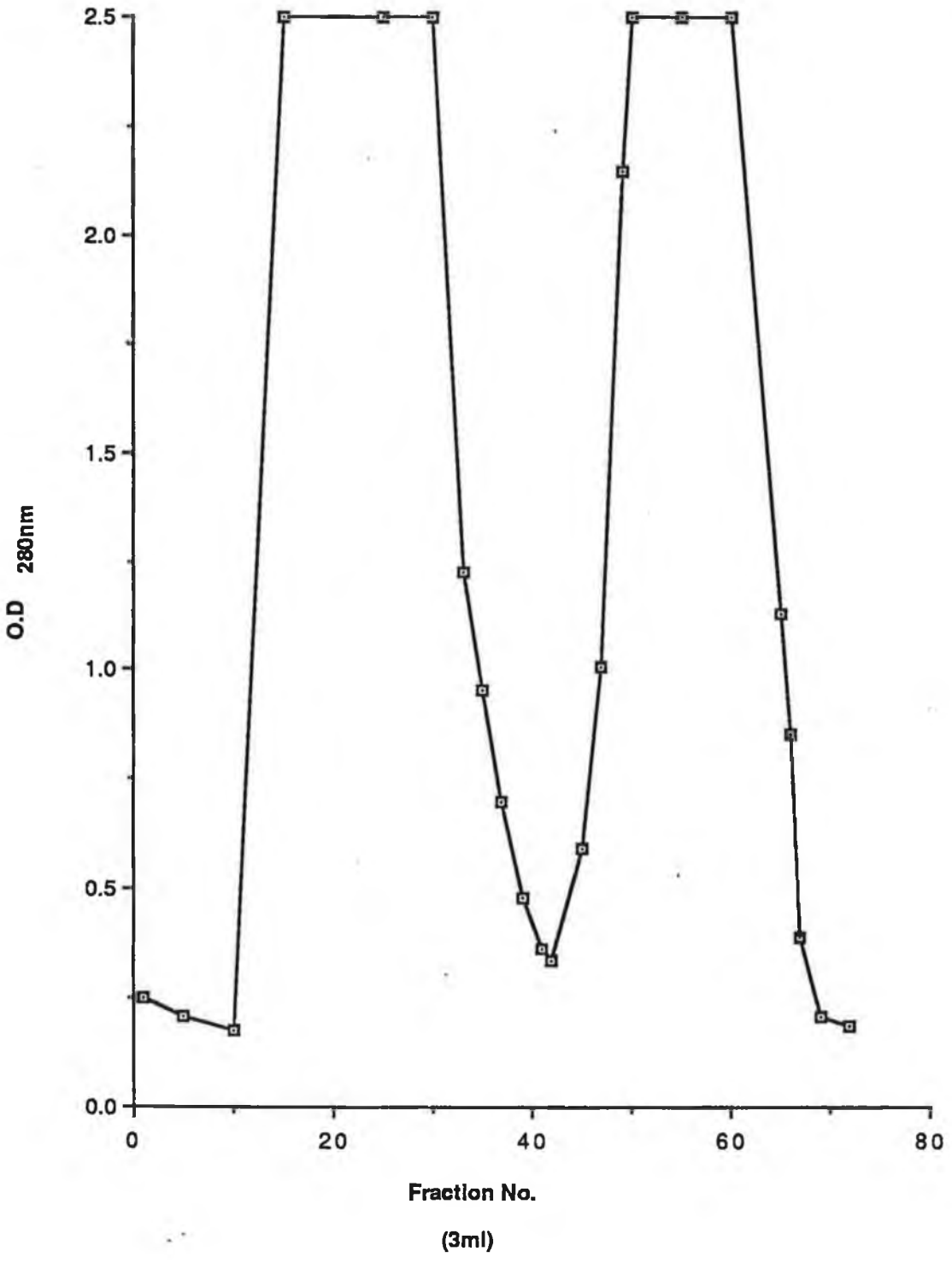

Figure 3.1.1 Sephadex G-75 chromatography of rat liver 105,000 g supernatant. Protein profile is illustrated by plotting O.D. 280nm Vs. fraction number.

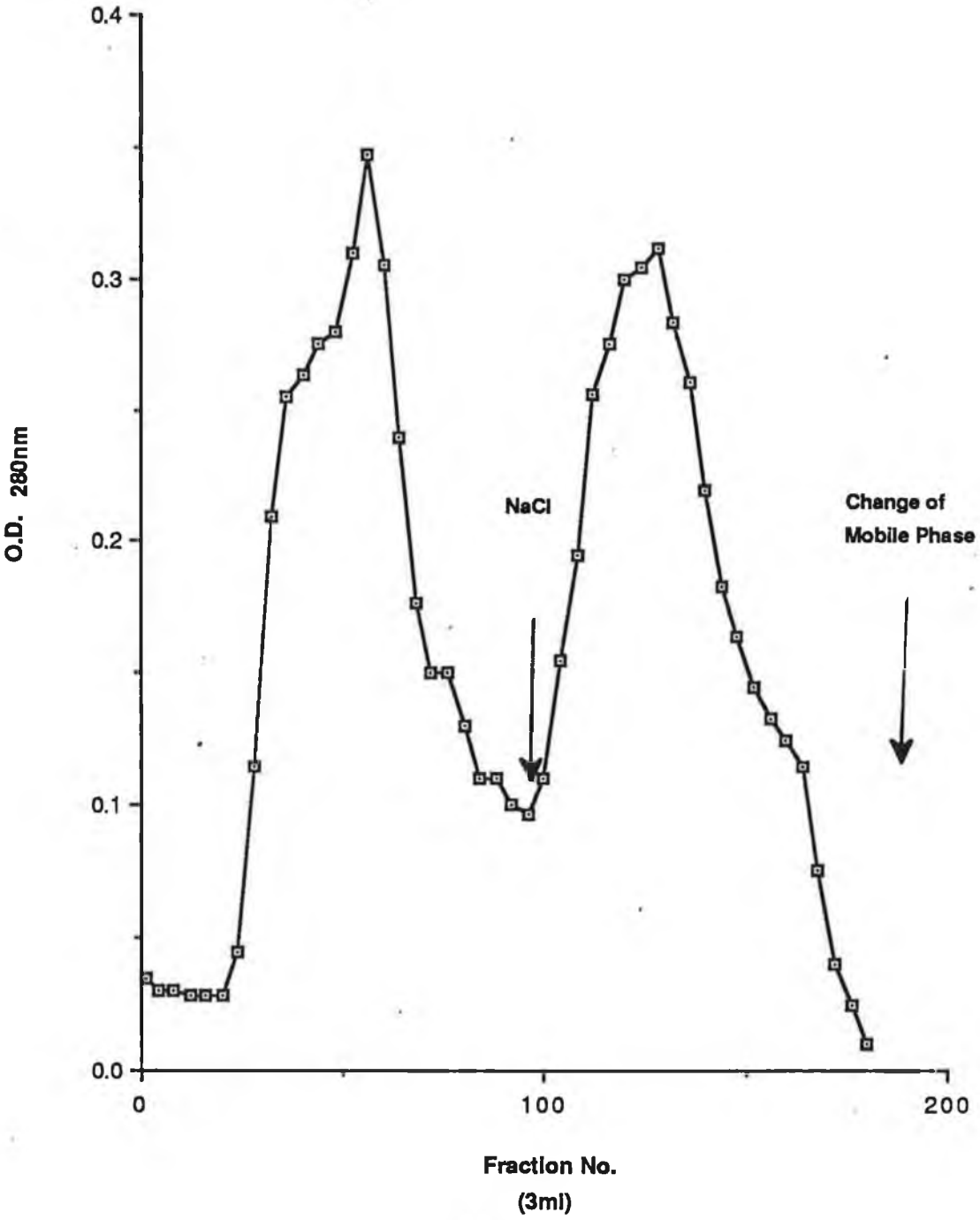

Figure 3.1.2 DEAE - cellulose chromatography of Sephadex G-75 Peak II protein. Protein profile is illustrated by plotting O.D. 280nm Vs. fraction number.

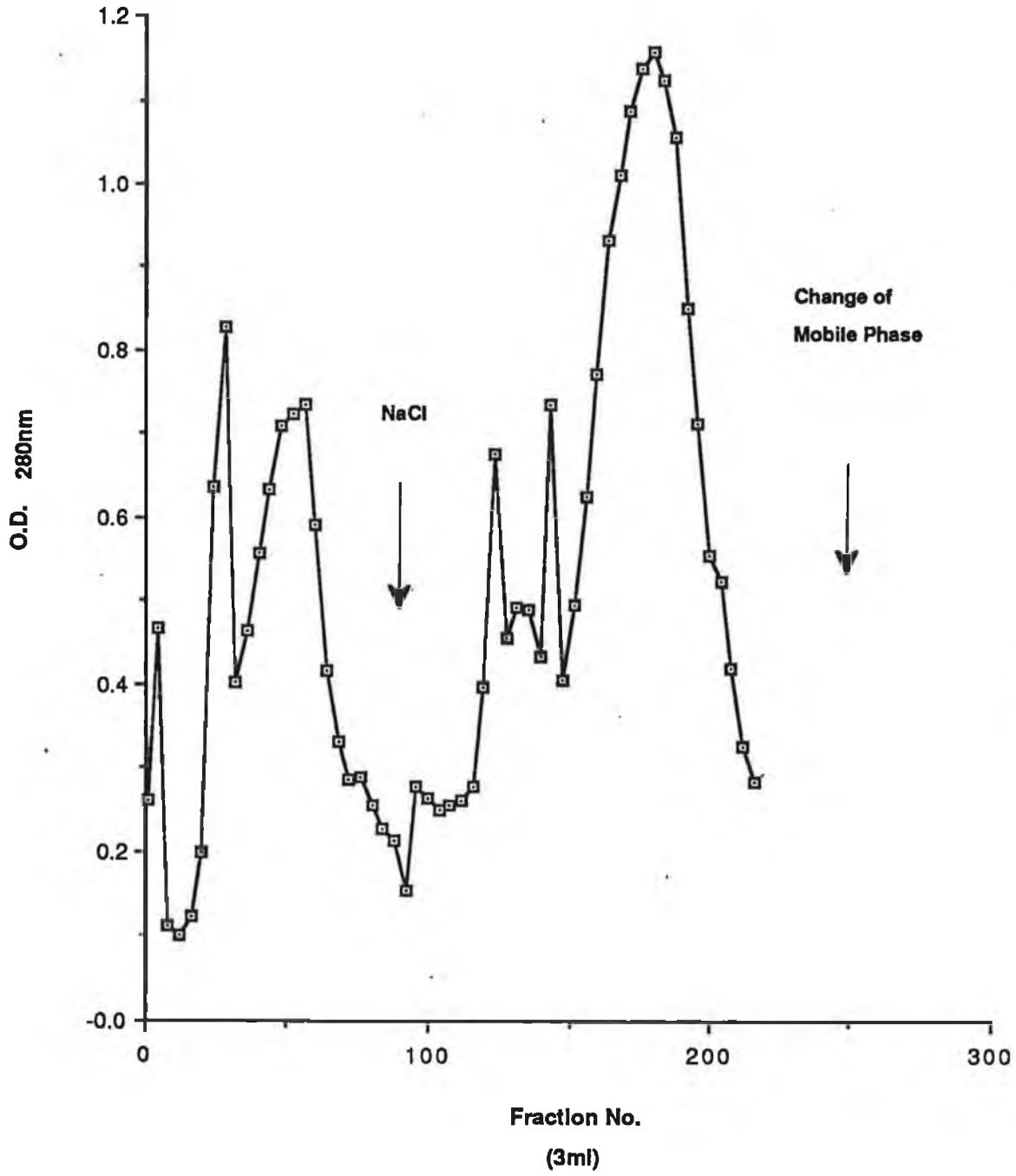

Figure 3.1.3 DEAE - cellulose chromatography of Sephadex G-75 Peak II protein

Protein profile is illustrated by plotting O.D. 280nm Vs. fraction number.

TABLE 3.1.1 **Protein content of different fractions collected during purification as determined by the Lowry method.**

PURIFICATION OF RAT LIVER SCP

FRACTION	VOLUME (ml)	TOTAL PROTEIN (mg)	PROTEIN YIELD (%)
105,000g Supernatant	10	1,600	100
G-75 Peak II Combined Fractions (42-68 Fig. 3.1.1)	120	58.8	3.7
DEAE-Peak I Combined Fractions (30-68 Fig. 3.1.2)	114	12.54	0.78
DEAE-Peak II Combined Fractions (104-150 Fig. 3.1.2)	138	36.8	2.3

The purity of the final protein peaks was checked by SDS polyacrylamide gradient gel electrophoresis (Figure 3.1.4). A sample of purified SCP (obtained as a donation from Dr. Mary Dempsey, Minneapolis, U.S.A.) was also run on the gel. The DEAE-Peak II sample, which was thought to be pure SCP, had one major band which co-migrated exactly with the donated SCP. However, a very fine band of protein (approximately 30,000 Daltons) was also visible in the DEAE Peak II material. It was thought that this might be an aggregate of SCP following a report by Dempsey et al., (1981) that some irreversible aggregation of SCP occurs during lyophylization. It should be noted however, that the donated SCP was also lyophylized prior to electrophoresis.

The DEAE-Peak I material showed two bands with slightly greater mobility than the SCP. In addition a band corresponding to that of SCP was also apparent in the lane comprising DEAE-Peak I material. This was not totally unexpected as Dempsey et al., (1981) reported that a low level of contamination of DEAE-Peak I with SCP does occur during purification and that rechromatography on an ion-exchange column completely resolves SCP from DEAE-Peak I protein. Similar observations were made by this author.

The molecular weight of proteins can be determined by comparison of their electrophoretic mobility in SDS polyacrylamide gradient gels with those of protein standards of known molecular weight. From the resultant standard curve (Figure 3.1.5) the molecular weight of the donated SCP and the purified SCP (DEAE-Peak II protein) was determined to be approximately 16,400 Daltons while that of the major DEAE-Peak I protein was found to be approximately 13,300 Daltons. Both these results correlate well with reported findings of Dempsey et al., (1981) where the values obtained were 16,000 and 14,000 Daltons respectively.

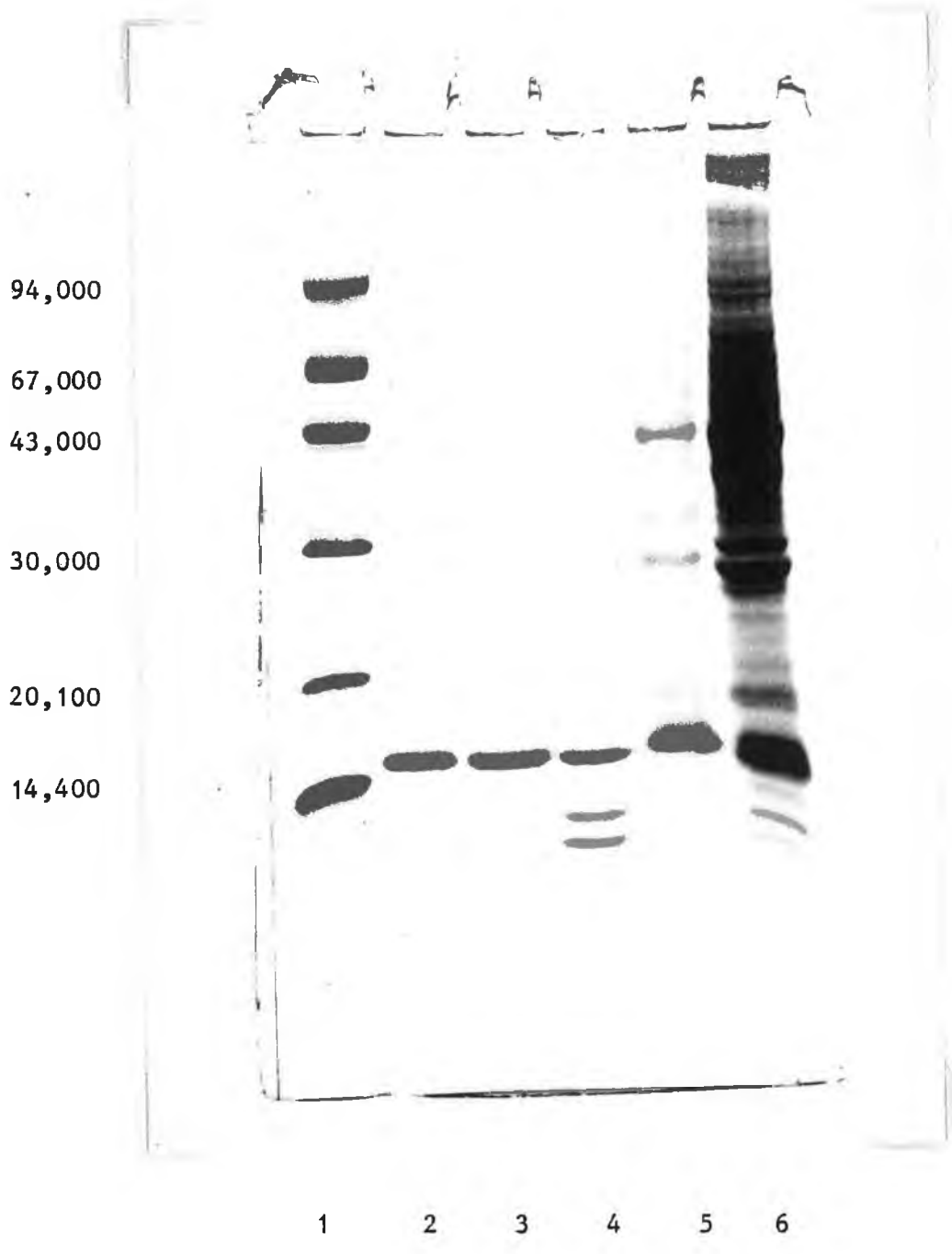

FIGURE 3.1.4 polyacrylamide gradient gel electrophoresis of different stages of SCP purification. Lane 1: Low molecular weight markers; Lane 2: donated SCP; Lane 3: purified SCP (DEAE-Peak II); Lane 4: DEAE-Peak I; Lane 5: Sephadex G-75 Peak II; Lane 6: 105,000g rat liver supernatent.

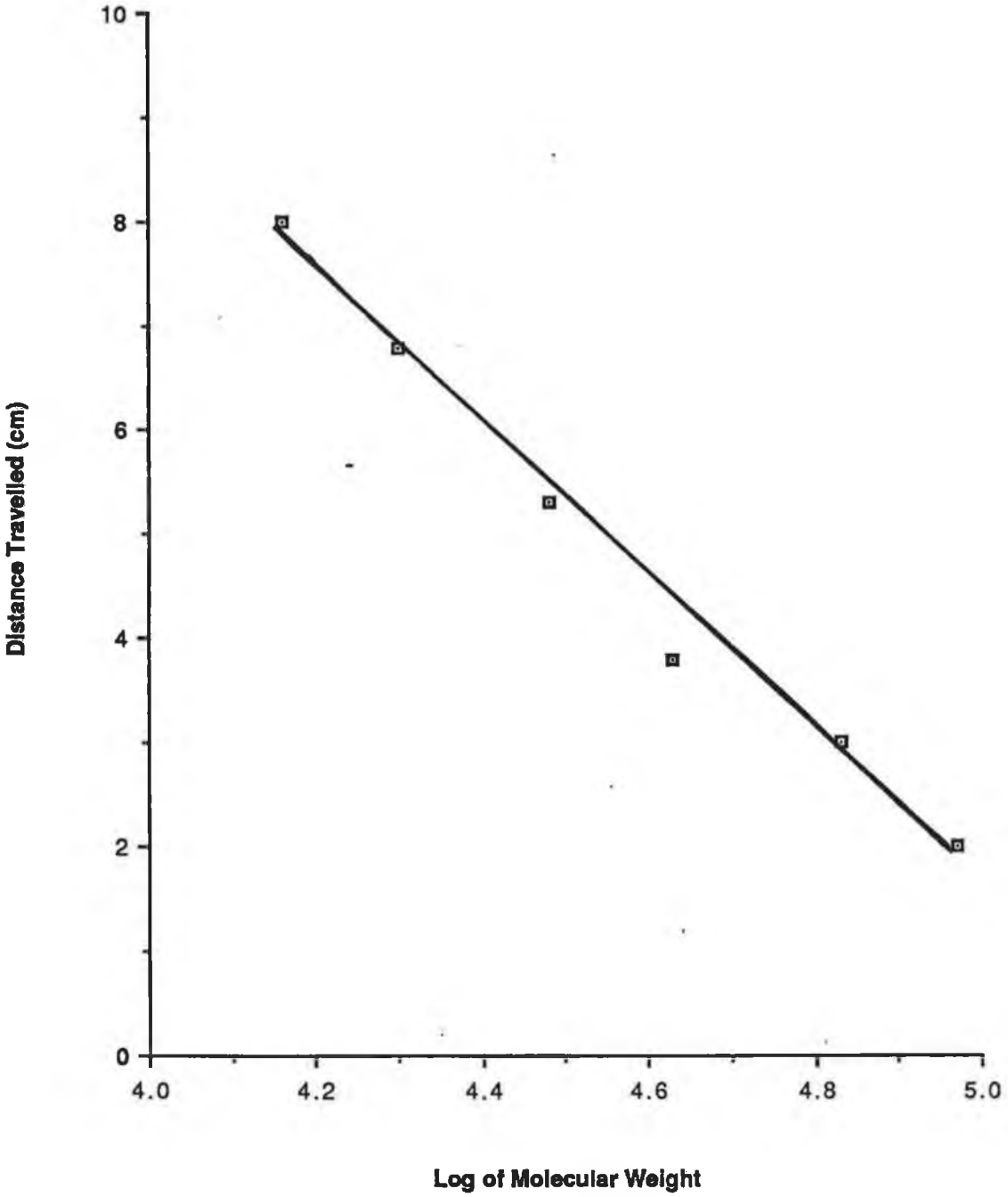

Figure 3.1.5 **Plot of the log of molecular weight of standard proteins against electrophoretic mobility in polyacrylamide gradient gels. Each point being determined by the mean of 12 estimations.**

3.2 Studies on Cholesterol and Fatty Acid Binding by SCP.

There is much controversy in the literature over the lipid binding properties of the protein known as SCP. It was thus decided to clarify the cholesterol and fatty acid binding properties of pure SCP. To demonstrate binding of ligands by binding proteins an efficient means of separation of bound and free ligand is required. A number of methods were tested in this study.

3.2.1 Cholesterol Binding Studies

3.2.1.1 Studies with Lipidex 1000 as the Separating Agent

Lipidex 1000 a 10% (w/w) substituted hydroxyalkoxypropyl derivative of Sephadex G-25 can be used to separate unbound and protein bound small hydrophobic molecules in a temperature dependent manner (Dahlberg et al., 1980). This property was exploited by Glatz and Veerkamp (1983) when they used Lipidex 1000 to demonstrate fatty acid binding by proteins.

Initially studies were carried out to show that a Lipidex 1000 column could be used to separate unbound and protein bound cholesterol in an aqueous solution. An aliquot of cholesterol solution (470 pmol/ml; 9.6 Ci/mmol) containing 141 pmol cholesterol was applied to a Lipidex 1000 column maintained at 4°C or 30°C. The first peak in Figures 3.2.1.1 and 3.2.1.2 represents the unbound cholesterol which was not retained by the column. This amounted to 21% and 19% of the labelled cholesterol applied to the column at running temperatures of 4°C and 30°C, respectively. Hence, approximately 80% of the total cholesterol applied was bound at both running temperatures. All of the bound cholesterol could be recovered from the column matrix by changing the mobile phase to methanol. The proportion of added cholesterol which did not bind was not reduced by increasing the column bed column or by reducing the amount of cholesterol applied.

The aqueous solution of labelled cholesterol was then incubated with BSA (400µg BSA with 94 pmol [^{3}H]- cholesterol) at 37°C

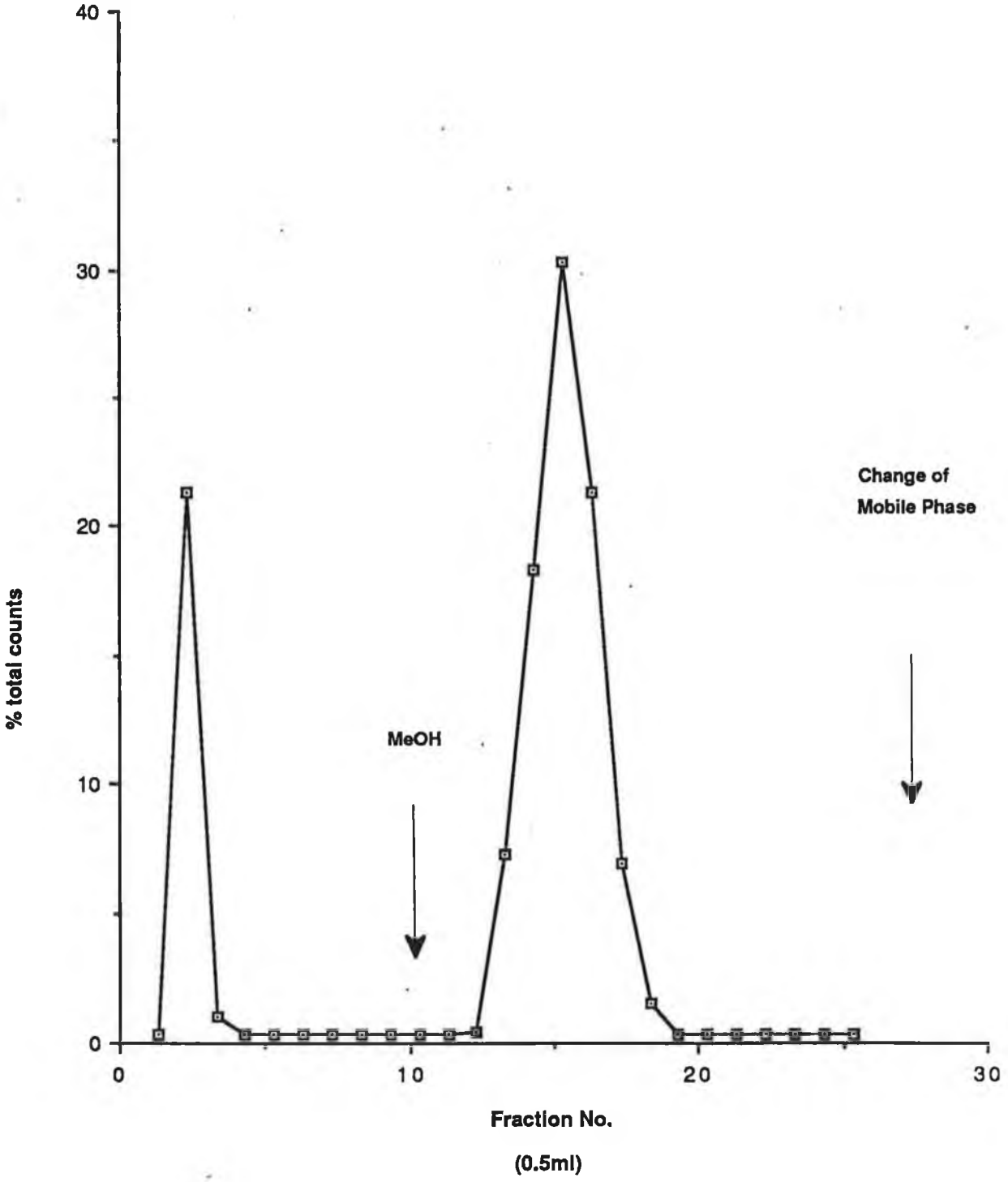

Figure 3.2.1.1 Elution profile obtained when 141 pmol [^{3}H] -cholesterol was applied to a Lipidex 1000 column at 4 ^{0}C

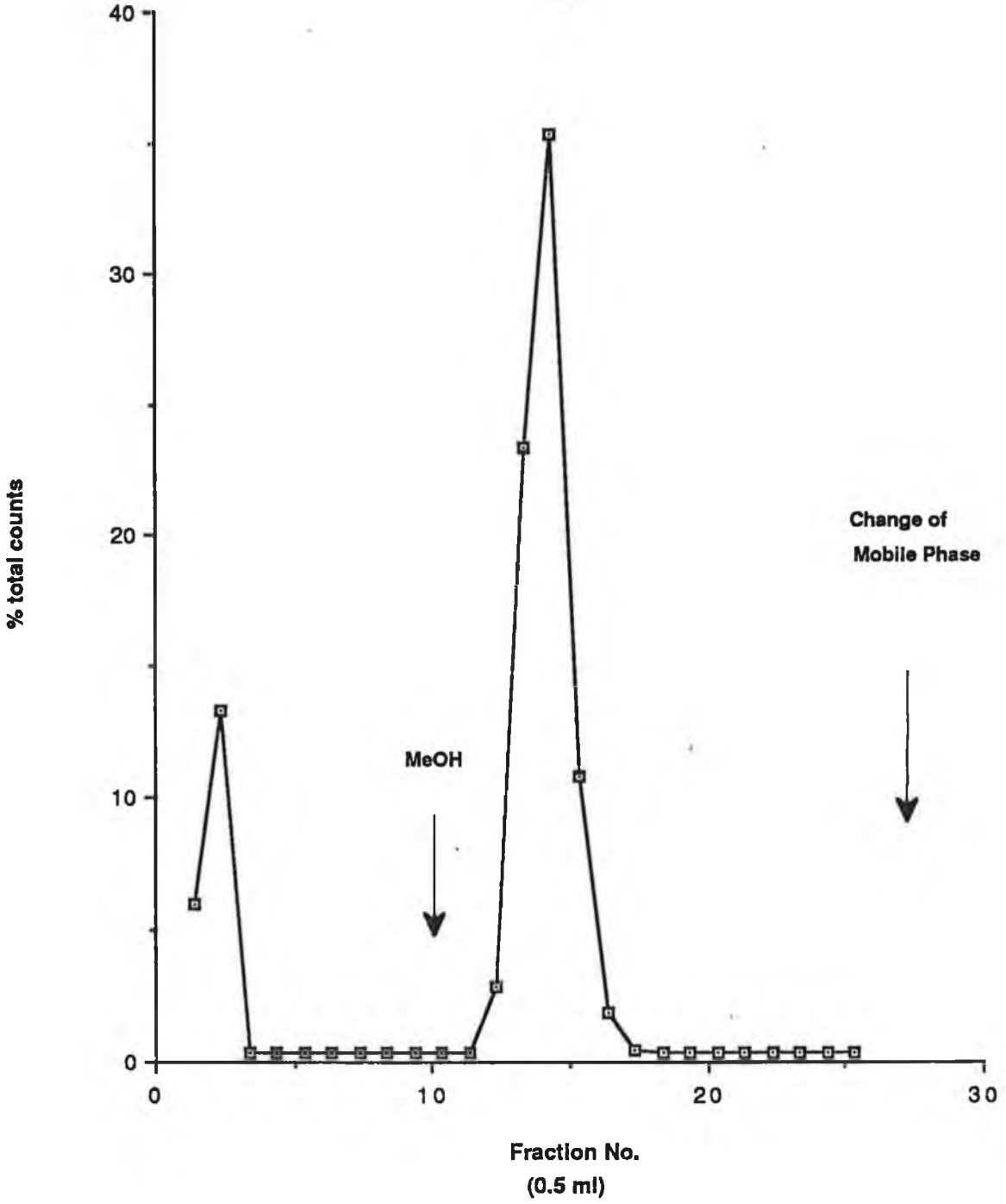

Figure 3.2.1.2 Elution profile obtained when 141pmol [^{3}H] - cholesterol was applied to a Lipidex 1000 column at 30 ^{0}C.

for 30 minutes before application to a Lipidex 1000 column which was being maintained at either 30°C or 4°C. At 4°C only 67% of the labelled cholesterol bound to the Lipidex 1000 Column, which was 12% less than that bound in the absence of BSA. This portion presumably represented BSA bound cholesterol (0.028 pmol/μg BSA). While the amount of cholesterol bound by BSA is low, the binding is still easily detectable using a Lipidex 1000 column. At 30°C, the percentage binding of cholesterol to the column was 82% which equals the percentage bound in the absence of protein. This suggests that at this temperature all BSA bound cholesterol was removed by the column (Figure 3.2.1.3) as already reported for protein bound fatty acids (Glatz and Veerkamp, 1983). Thus it appeared that a column of Lipidex 1000, at 4°C, was suitable for the separation of protein bound and free cholesterol. However, prior incubation of purified SCP with the labelled cholesterol did not lead to any change in the elution profile of the column, when run at 4°C (Figure 3.2.1.4). Therefore, the concentration of free label was not affected by the presence of SCP, which indicates that SCP did not bind any cholesterol during the incubation step.

3.2.1.2 Studies with Activated Charcoal as the Separating Agent

Charcoal is known to adsorb small hydrophobic molecules in a time and temperature dependent manner and is commonly used in binding studies to separate bound from free ligand. As a large number of different protocols for charcoal have been reported, it was first necessary to establish those conditions which would be optimum for separation of bound and free cholesterol. Various charcoal preparations were examined as shown in Table 3.2.1.1. The presence of Dextran and/or gelatin was found to reduce, substantially, the binding of free label by charcoal and hence increase non-specific binding. It was found that minimum non-specific binding occurred when a simple suspension of 0.7% (w/v) charcoal in PBS (pH 7.4) was used.

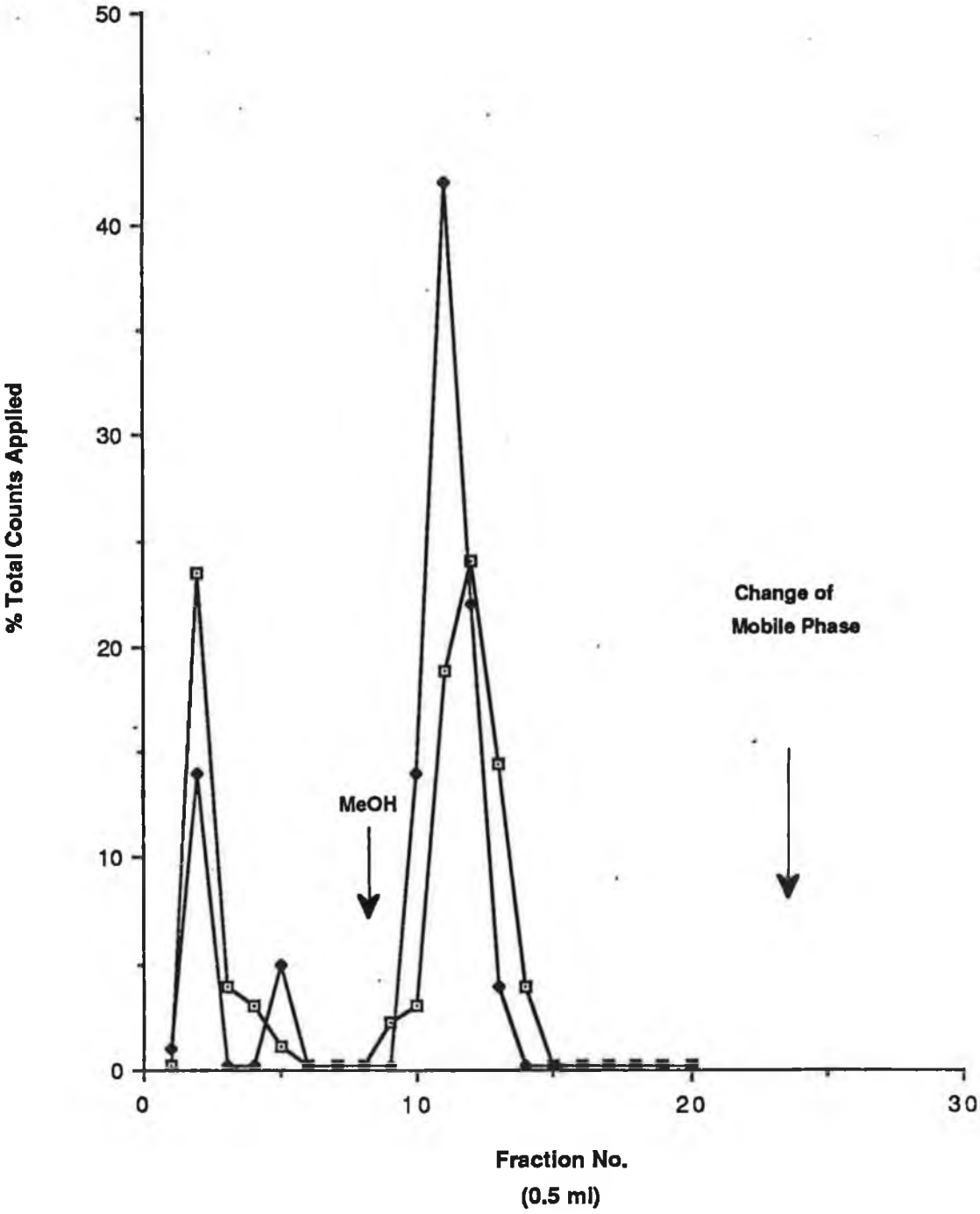

Figure 3.2.1.3 Elution profile obtained when a mixture of 400ug BSA and 94 pmol [^{3}H] - cholesterol was applied to a lipidex 1000 column at 4 ^{0}C ( ) and 30 ^{0}C ( ) BSA and labelled cholesterol were incubated at 30 ^{0}C for 30 minutes before applying to column.

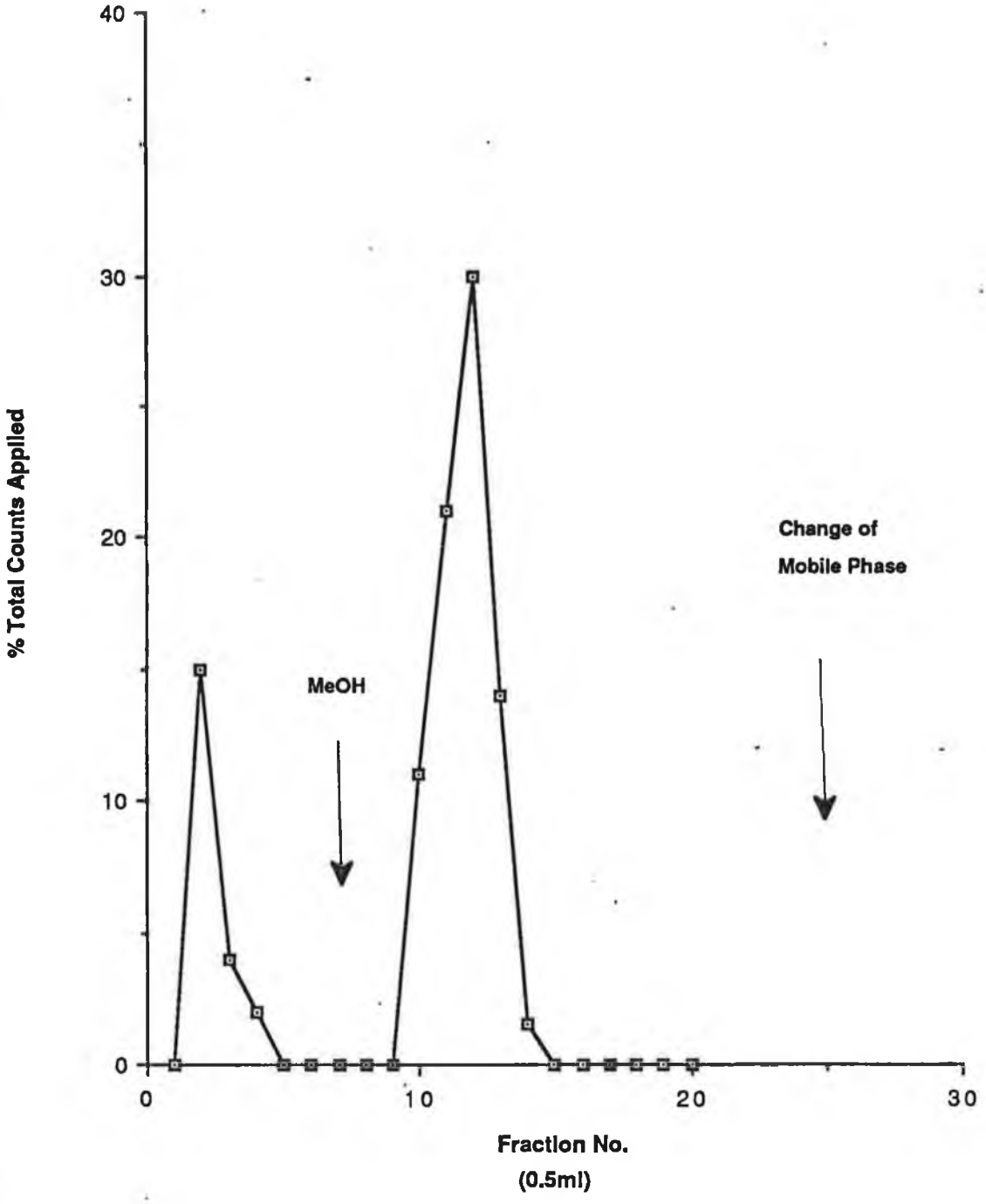

Figure 3.2.1.4 Elution profile obtained when a mixture of 8.6ug SCP and 47 pmol [^{3}H] - cholesterol was applied to a Lipidex 1000 column at 4 ^{0}C. SCP and labelled cholesterol were incubated at 37 ^{0}C for 30 minutes before application to the column.

TABLE 3.2.1.1. Effect of the presence of dextran and gelatin on the binding of free [^{3}H] - cholesterol by 0.7% (w/v) charcoal suspensions in PBS.

Charcoal Preparation	Binding of Label to Charcoal %	Non-Specific Binding of Label %
0.7% Charcoal	74	26
0.7% Dextran Coated Charcoal	67	33
0.7% Charcoal and 0.1% Gelatin	49	51
0.7% Dextran Coated Charcoal and 0.1% Gelatin	39	61

To further reduce non-specific binding the percentage charcoal was then varied from 0.1-1% (w/v). This resulted in a continued decrease in non-specific binding of label as the percentage of charcoal used was increased. (Table 3.2.1.2). The results reported represent an average of 10 identical experiments. Because of difficulty encountered with pelleting of the charcoal when it was used at greater than 0.7% (w/v) the less concentrated suspension of charcoal, 0.7% (w/v), was chosen for subsequent studies.

The cholesterol binding capacity of SCP was then investigated by incubation of the label (0.76 pmol [^{3}H]-cholesterol; 32.9 Ci/mmol) with varying amounts of the protein and subsequent separation of the free ligand by charcoal treatment. The presence of SCP (0.1μg-100μg/assay tube) caused no change in the concentration of free cholesterol measured, thus suggesting that no binding of cholesterol by SCP had occurred. However, BSA at a concentration of 10 μg/assay tube (the concentration used by Glatz and Veerkamp (1983) in palmitate binding studies) was found to bind 0.06 pmol of [^{3}H]-cholesterol. This is equivalent to 0.006 pmol/μg BSA. The lower degree of binding observed using this method was not totally unexpected as Glatz and Veerkamp, (1983) reported that separation of protein-bound and free fatty acids by charcoal resulted in a lower apparent degree of protein-binding than found with Lipidex 1000. This arises due to stripping of the protein-bound ligands by charcoal. Nevertheless, binding of cholesterol by BSA was clearly demonstrated by this method thereby lending support to the lack of cholesterol binding activity by SCP, regardless of the concentration tested. Prior delipidation of the SCP preparation as outlined in Materials and Methods (Section 2.2.2.3) did not affect the results.

3.2.1.3 Use of Gel Filtration in Separation of Bound and Free Ligand

Ritter and Dempsey (1973) have reported cholesterol binding by a partially purified preparation of SCP. They used gel filtration to separate SCP bound from free cholesterol and it was decided to attempt to repeat this experiment with the SCP

TABLE 3.2.1.2 **Binding of [^{3}H]-cholesterol to charcoal present at varying concentrations**

Charcoal Concentration	Binding of [^{3}H]-Cholesterol to Charcoal	Non Specific Binding of [^{3}H]-Cholesterol
%	%	%
0.1	0	100
0.3	66	34
0.5	69	31
0.7	74	26
0.8	80	20
0.9	81	19
1.0	85	15

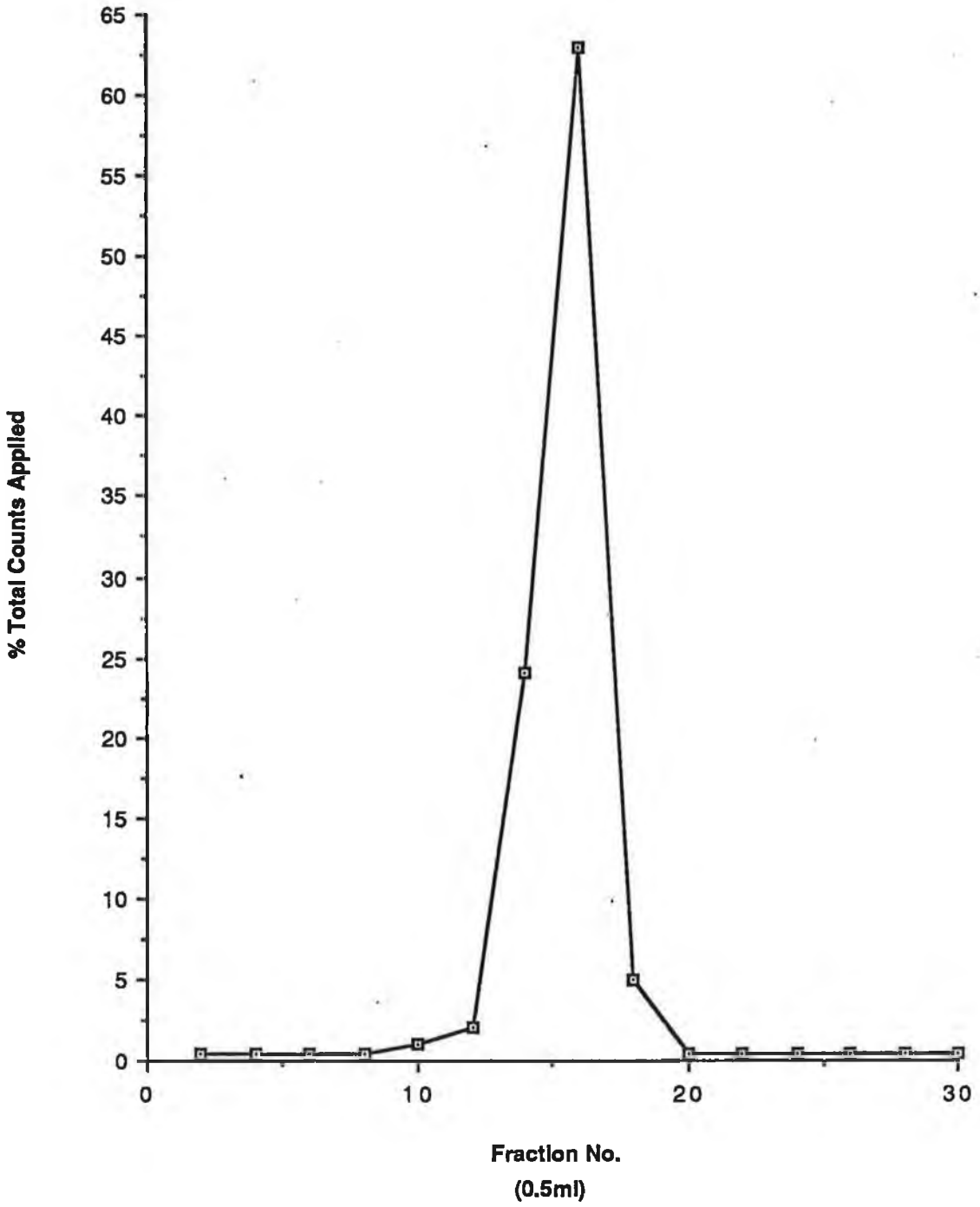

Figure 3.2.1.5 Typical elution profile of [^{3}H] - cholesterol following application to a Sephadex G-25 gel filtration column.
Void volume of the column was 9.3ml.

of higher purity prepared during this study. An aqueous solution of labelled cholesterol (7.6 pmol/ml) with a specific activity of 32.9 Ci/mmol, was applied to Sephadex G-10, G-25 and G-75 gel filtration columns. However, in all cases the label was unexpectedly eluted in the void volume (Figure 3.2.1.5 depicts a typical profile obtained). Because of its low molecular weight, it was expected that free cholesterol would be retained by these columns. The results obtained indicated that the cholesterol molecules had aggregated to form a higher molecular weight structure. The critical micellar concenration of cholesterol is reported to be 25-40 nM (25-40 pmol/ml) at 25°C (Haberland and Reynolds, 1973) and to decrease with decreasing temperature. It is possible, therefore, that at room temperature, 10-12°C, a 7.6 pmol/ml solution of cholesterol would exist as micelles, which would then appear in the void volume.

Since Ritter and Dempsey (1971) used a propylene glycol solution of cholesterol in their studies rather than an aqueous solution, a repeat of their experimental procedure was attempted. When the [^{3}H]-cholesterol solution was prepared in propylene glycol, as described in Section 2.2.2.1 and applied to each of the individual columns the label was again eluted in the void volume (Figure 3.2.1.6 depicts a typical profile obtained). Therefore, aggregation of cholesterol must also occur in this solvent. In all cases, approximately 95% of the label applied to the column was recovered. Therefore, gel filtration was found to be unsuitable for the separation of protein bound and free cholesterol.

3.2.2 Fatty Acid Binding Studies

There is less dispute in the literature over the fatty acid binding property of the protein known as SCP. It was decided to investigate the fatty acid binding property of the SCP prepared in this study. Since charcoal is the most frequently used method of separation for protein-bound and free ligands; it was chosen for use in the separation of SCP-bound and free oleic acid. A similar protocol was followed as was used in the cholesterol-binding studies.

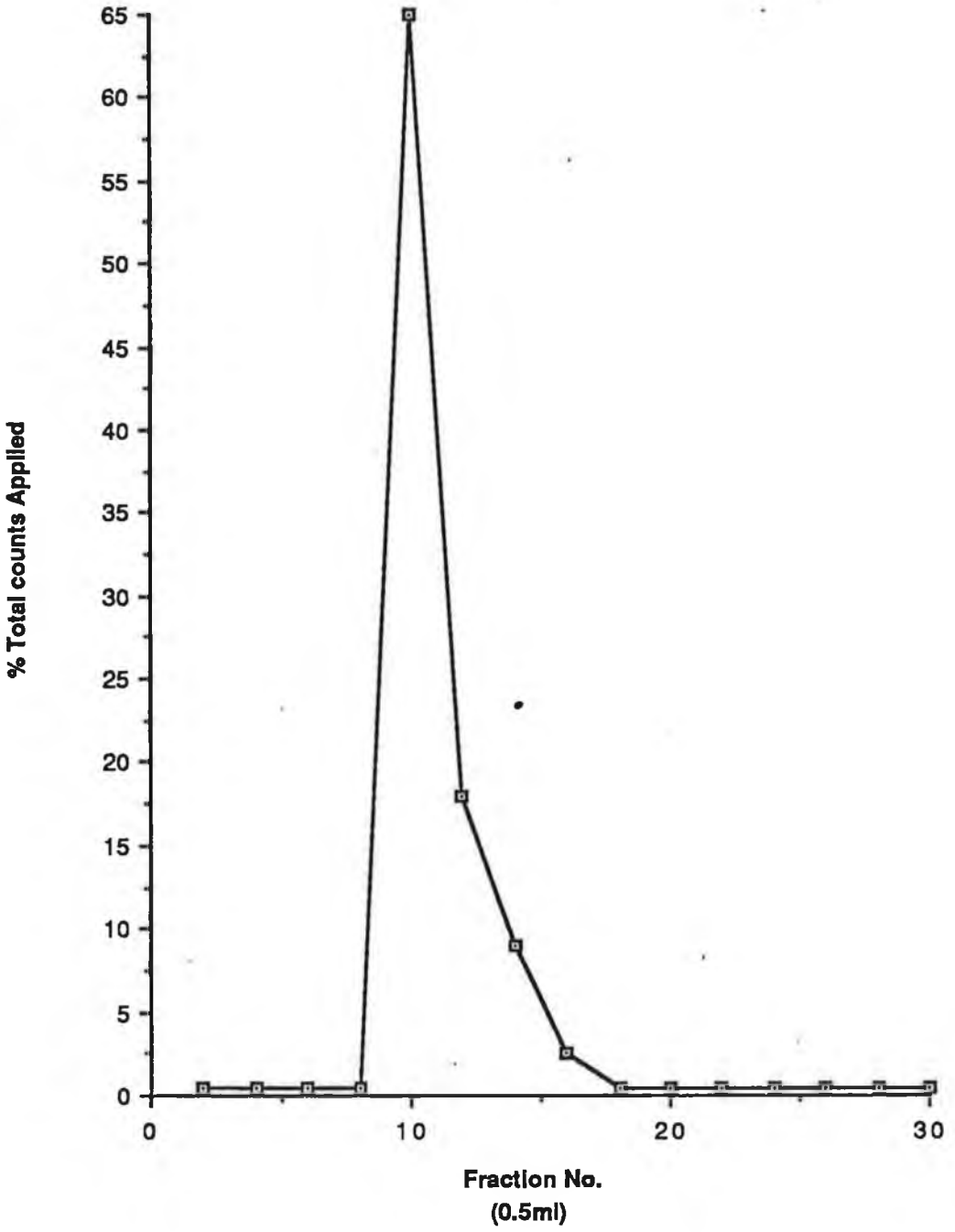

Figure 3.2.1.6 Typical elition profile of [^{3} H] - cholesterol in propylene glycol following application to a Sephadex G-25 gel filtration column. Void volume of the column was 9.3ml.

In the absence of protein, 82% of [^{3}H]-oleic acid was bound by the charcoal suspension (0.7% (w/v) in PBS buffer (pH 7.4). This implies 18% non-specific binding of the label under the experimental conditions used. The recovery of free fatty acid was substantially reduced by prior incubation of the labelled fatty acid with both SCP and BSA indicating that both of these proteins have fatty acid binding capabilities. 10µg BSA was found to bind 15 pmoles [^{3}H]-oleic acid while 10µg SCP was found to bind 12 pmoles [^{3}H]-oleic acid. This is equivalent to 1.5 pmol/µg BSA and 1.2 pmol/ µg SCP. It seems, therefore, that the protein purified earlier in this study was indeed capable of binding fatty acids, but this study has failed to show cholesterol-binding activity by this protein.

3.3 Antibodies to SCP

3.3.1 Antibody Titre Determination

Antibodies to both native SCP and to the SCP band cut from the SDS polyacrylamide gradient gels were raised in both female Balb C mice and New Zealand White rabbits as outlined in sections 2.2.3.1 and 2.2.3.2, respectively. With respect to the rabbits, bleeds were taken at regular intervals to monitor antibody production. The final bleed, taken following boosting, was subjected to immunoglobulin enrichment as described in section 2.2.3.3. In the case of the mice ascites fluid was collected and stored until no more was produced and then it, too, was enriched for immunoglobulin.

A sandwich type ELISA was used in titre determination. Wells of the microtitre plates were coated with 10 µg/well SCP - estimated to be a large excess - washed well and then reacted with varying dilutions of the test antisera (section 2.2.3.4). After washing the wells they were reacted with the appropriate second antibody conjugated to the enzyme;- horse radish peroxidase (HRP) - donkey anti-mouse IgG-HRP and mouse anti-rabbit IgG-HRP. The dilution factor recommended by the manufacturers for each of these conjugates was found to be too high. Thus a suitable working dilution was determined as being that which yielded adequate colour development with the test antisera. A dilution of 1 in 2000 was found to be suitable for

the donkey anti-mouse IgG-HRP conjugate. However, problems were encountered with the stability of the commercial mouse anti-rabbit IgG-HRP conjugate. Initially the enzyme activity was found to be unstable and activity was greatly reduced both on storage at 4°C and at -20°C. In replacement batches the antibody binding activity of the commercial conjugate was found to be unstable while the enzyme maintained its activity. An alternative conjugate was kindly donated by the Department of Biochemistry, University College, Galway, which proved to be more stable and a working dilution of 1 in 400 was established for this conjugate.

Antibody titres of rabbit bleeds were determined as outlined earlier. The titre was taken as being that dilution which gave an $O.D_{490nm}$ of 1.0. The titre was established by plotting the $O.D_{490nm}$ against serial dilutions of the respective antisera. A typical plot is illustrated in Figure 3.3.1. The antibody titres for the remaining living rabbits before and after boosting are outlined in Table 3.3.1.

Large bleeds were taken from all rabbits except R_{280} which showed a poor antibody response. The immunoglobulin fraction was partially purified from each bleed (section 2.2.3.3) for further characterization. Both groups of mice yielded sufficient ascites for further characterization - M_{100} which were immunized with native SCP and M_{40} which were immunized with polyacrylamide-gel-incorporated SCP. The total ascites collected from each group was pooled and also subjected to immunoglobulin enrichment before characterization. The resulting enriched immunoglobulin fractions were then assayed to determine their respective antibody titres. Titres for both ascites samples were low 90 and 990 for M_{42} and M_{100}, respectively (Table 3.3.2). Since the volume of enriched IgG recovered was also low in both these cases, these samples were not subjected to any further characterization. Titres for rabbits range from 75 for R_{262} which was immunized with PAG-incorporated SCP to 7,800 for R_{289} which was immunized with native SCP (Table 3.3.2).

This study aimed to produce two antisera with high activity towards native SCP. These would enable the development of a

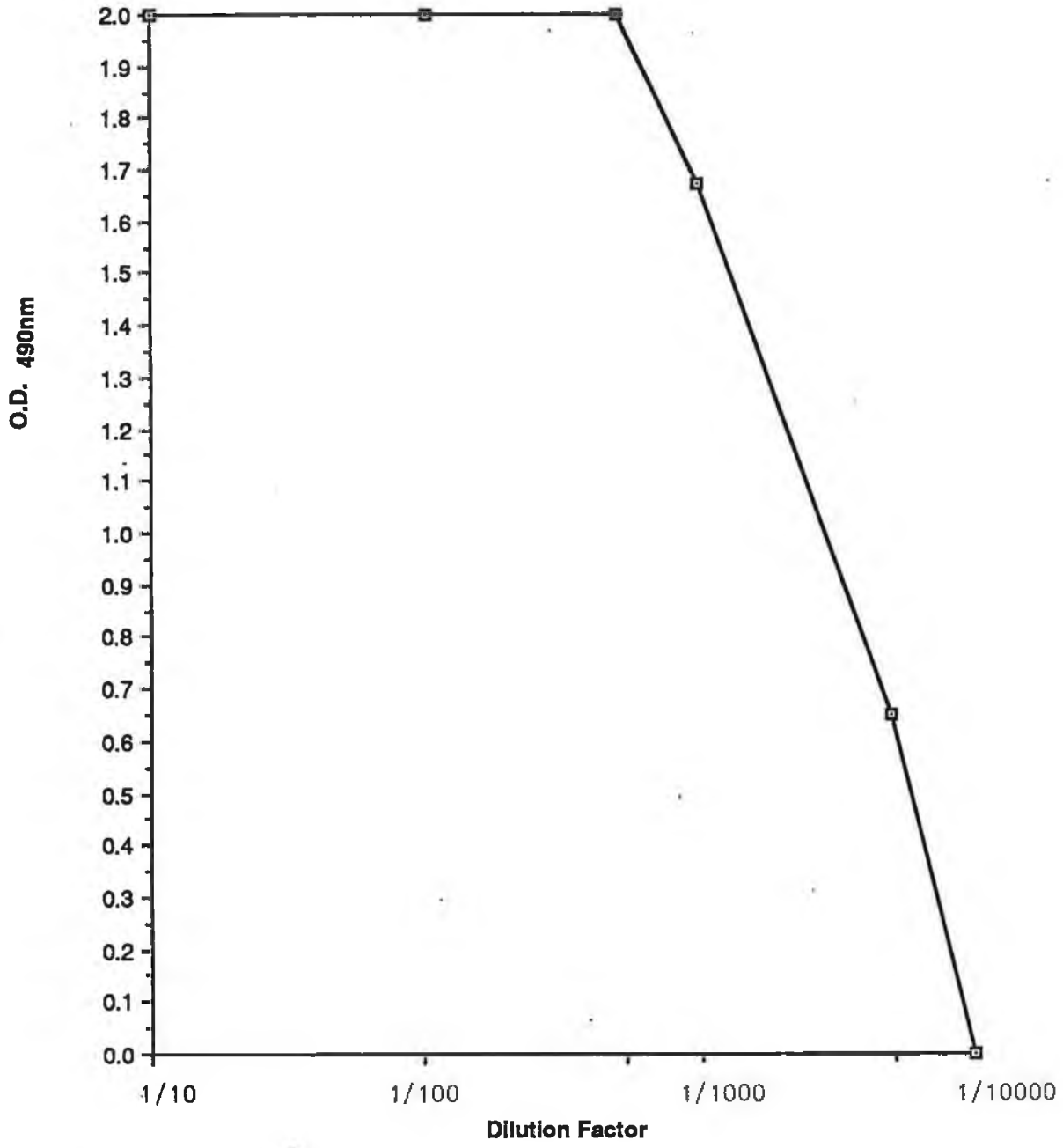

Figure 3.3.1 Antibody titre determination.
Graph depicts a typical profile obtained by plotting the O.D 490nm Vs. diluted antisera.

TABLE 3.3.1 Summary of rabbit anti-SCP Titres pre and post-boosting

Animal	Immunogen	Route of Immunization[b]	Maximum Titres of Rabbit Bleeds[a]	
			Pre-Boost	Post-Boost
R_{262}	PAG-incorporated SCP	I.D.	325	880
R_{279}	" " "	I.D.	460	2,060
R_{280}	" " "	I.D.	95	435
R_{282}	Native SCP	S.C.	880	2,600
R_{284}	" "	I.D.	1,125	3,000
R_{289}	" "	I.D.	1,330	3,500

(a) Titres are expressed as the reciprocal of the dilution factor giving an O.D. 490 of 1.0.

(b) I.D. - Intradermal injection
S.C. - Subcutanous injection

TABLE 3.3.2 Anti SCP titres of immunoglublin enriched ascites and antisera

ANIMAL	IMMUNOGEN	ROUTE OF IMMUNIZATION	VOLUME OF ENRICHED Ig (ml)	TITRE
M_{42}	PAG-incorporated SCP	I.P. *	0.5	90
M_{100}	Native SCP	I.P. *	0.5	990
R_{262}	PAG-incorporated SCP	I.D. *	2.5	75
R_{279}	PAG-incorporated SCP	I.D. *	2.75	200
R_{282}	Native SCP	S.C. *	3.0	470
R_{284}	Native SCP	I.D. *	4.0	6,600
R_{28}	Native SCP	I.D. *	4.2	7,800

* Route of immunization·

I.P. - intraperitoneal
I.D. - intradermal
S.C. - subcutaneous

sensitive sandwich enzyme immunoassay for SCP in biological systems (i.e. native SCP). Thus the antisera from the two rabbits R_{284} and R_{289}, which were immunized intradermally with native SCP, were selected for further characterization.

3.3.2 Antibody Specificity

It was necessary to determine the specificity of antisera R_{284} and R_{289}. The major expected cross reactants were the DEAE-Peak I protein, which co-purified with SCP up to a certain stage of the purification procedure, and albumin, which is the major extracellular fatty acid binding protein and hence might share common antigenic determinants with SCP. Cross reactivity towards gelatin was also determined. Wells of a microtitre plate were coated with these proteins at two concentrations, 10 and 100 µg/well and the colour obtained compared with that obtained with SCP coated at 10µg/well. Results indicated that neither antisera R_{284} nor R_{289}were active against DEAE-Peak I protein or gelatin. In addition the antisera from R_{284} was not found to show any reaction to BSA while R_{289} showed a slight response against BSA, equivalent to 4.5% cross reaction (Table 3.3.3).

3.3.3 Antibody Sensitivity

For the determination of antibody titres the wells of microtitre plates were each coated with 10µg of purified SCP (Section 2.2.3.4). In selecting this coating concentration, it was assumed that this quantity of SCP was an excess. It was necessary, therefore, to determine the sensitivity of the antisera R_{284} and R_{289} against SCP. Wells were coated with SCP concentrations which ranged from 0.1µg/ml to 10µg/ml (i.e. 0.02µg/well to 2µg/well). Both enriched IgG preparations were used at 1 in 5000 dilution for these experiments. With both antibody preparations, the colour response was found to increase as coating concentration increased from 1 to 10µg/ml (Figure 3.3.2). The degree of colour obtained with R_{289} was slightly higher than that obtained with R_{284} (Figure 3.3.2). At concentrations below 1µg/ml the colour produced was not significantly different from background with either of the IgG

TABLE 3.3.3. Reaction of antisera R284 and R289 against different proteins

PROTEIN SAMPLE	PROTEIN CONCENTRATION (g/well)	O.D.490 R_{284}	O.D.490 R_{289}
SCP	10	1.123	1.242
DEAE-Peak I	10	0	0
BSA	10	0	0.145
BSA	100	0	0.056
Gelatin	10	0	0
Gelatin	100	0	0

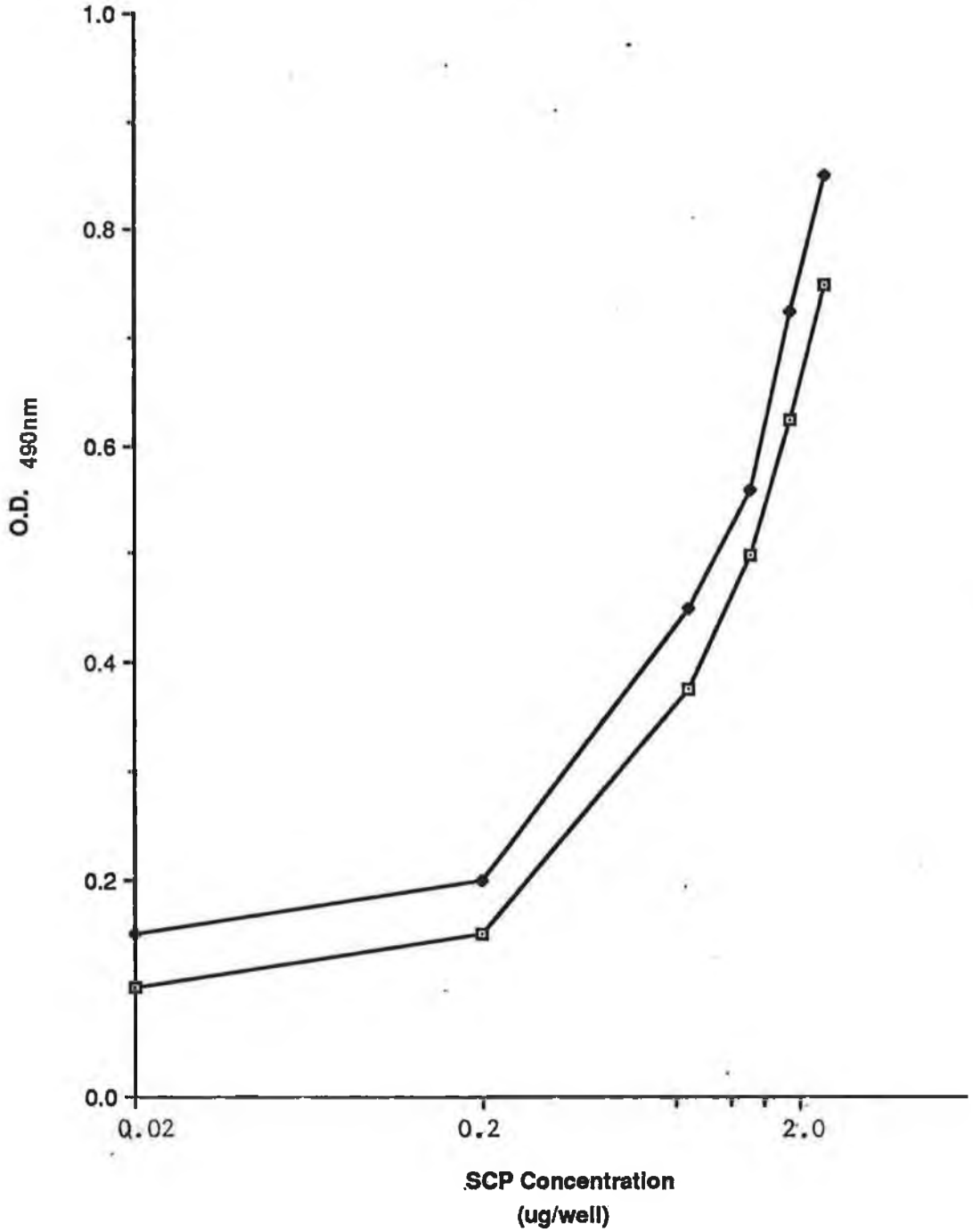

Figure 3.3.2 Detrermination of the sensitivity of the IgG preparations R_{284} ( ) and R_{289} ( ) toward SCP.

preparations.

These results indicate that both selected antisera can be used to detect 0.2μg of SCP and upwards when the protein is coated directly onto the plate. The coating step is, however, generally accepted to be less than 100% efficient. Therefore, in a sandwich assay where all SCP present in the sample would be trapped by a solid phase antibody to SCP, the detection limit would be expected to be lower than this value.

Thus this study has resulted in the production of two anti-SCP antisera which show sufficient specificity in their binding towards SCP. They can be used to detect SCP levels at least as low as 0.2μg and their titres are such that they are available in adequate quantities for development of a sandwich ELISA for SCP.

4 DISCUSSION

The object of this project was to purify SCP from rat liver; clarify its lipid binding properties and lay the ground work for a rapid and sensitive assay for SCP. This study was initiated as a result of the observation that SCP is also found in serum where it is mainly associated with the lipoprotein fraction (Dempsey et al., 1984a). It is, therefore, not solely an intracellular protein. It was of interest to investigate the mechanism of association between SCP and the various lipoproteins which it was thought might involve binding of SCP to cholesterol on the surface of the lipoprotein particle. In addition serum SCP levels have more recently been found to be elevated in individuals suffering from atherosclerosis (unpublished data). A rapid and sensitive assay would prove useful in further studies on serum levels of SCP. The assay system which has been used to date does not lend itself to rapid screening of large numbers of samples. Hence, this study also aimed to initiate the development of such an assay. For both of these objectives purified SCP was required, which was prepared in the first part of this study.

Purification of SCP was performed according to the the method of Dempsey et al., (1981). On all occasions, the protein profile obtained following gel filtration was identical to the reported observations of Dempsey et al., (1981). Many of the protein profiles following ion-exchange chromatography were also as reported by Dempsey et al., (1981). However, in some instances the protein, which bound to the column, formed two distinct peaks when eluted with NaCl; the smaller extra peak (DEAE-Peak IIa) was eluted prior to the major second peak (DEAE-Peak II or SCP). While no such peak has been reported in the literature pertaining directly to SCP, it has been observed that FABP, Z-protein and Protein A (Glatz and Veerkamp, 1985; Trulzsch and Arias, 1981; Takahashi et al., 1983 and Ketterer et al., 1976) gave a number of peaks following ion-exchange chromatography. These differences have been attributed to the presence or absence of specific fatty acids bound to the protein when isolated, which would ultimately affect the isoelectric point of the protein and thus its elution profile when subjected to ion-exchange chromatography. It was noted, in this study, that the smaller peak (DEAE-Peak IIa) occurred only when older animals were used as the source of the

protein, i.e. animals greater than 300 g body weight. Dempsey et al., (1981) only reported the use of animals between 200-250 g body weight. The different profile in older animals may be due to different distribution of fatty acids in the SCP of older animals.

Many of the reported functions of SCP, e.g. cholesterol synthesis, adrenal steroidogenesis would require an ability to bind sterol precursors and cholesterol itself. Ritter and Dempsey (1973) reported binding of cholesterol and its esters by a crude preparation of SCP. However, Scallen et al., (1985) could not attribute any sterol carrier protein activity to FABP (a protein believed to be identical to SCP). FABP was not capable of releasing cholesterol from adrenal lipid droplets nor did it stimulate the utilization of endogenous mitochondrial cholesterol for pregnenolone production. These assays were designed to mirror the physiological role of a carrier protein. No organic solvents were used (Scallen et al., 1985). In this study an attempt was made to clarify the sterol binding capacity of purified SCP using conventional binding assay methodology. No binding of cholesterol by SCP was observed during these studies regardless of the method used. However, BSA was found to bind cholesterol in aqueous solution using two different methods for separation of protein-bound and free ligand. The degree of binding was very low as expected, since physiologically albumin does not contribute significantly to cholesterol transport. The discrepancy in the results obtained by the two methods (0.028 pmoles cholesterol/ g BSA using Lipidex 1000 and 0.006 pmoles/ g BSA using charcoal) was not unexpected as Glatz and Veerkamp (1983) reported that Lipidex 1000 was much more effective in the separation of bound and unbound fatty acid than charcoal.

In 1973, when Ritter and Dempsey reported cholesterol binding by a crude preparation of SCP, they used gel filtration to separate unbound from protein-bound ligand. During this study, an attempt was made to repeat these results using purified SCP. However, initially the cholesterol was prepared in aqueous solution rather than propylene glycol to avoid introduction of an organic solvent which was considered to be non-physiological. It was found that regardless of the exclusion limit of the gel used (Sephadex G-75 3,000-70,000 Daltons; Sephadex G-25 100-5,000 Daltons and Sephadex G-10 0-700 Daltons) free cholesterol was eluted repeatedly in the

void volume. Such a finding was unexpected as cholesterol, having a molecular weight of 387 Daltons, should have been retained by each of the gels used. It has been reported that cholesterol undergoes thermodynamically reversible self-association in aqueous solution with a critical micelle concentration of 25-40 nM at 25°C (Haberland and Reynolds, 1973).

This concentration is higher than that used in the experiments reported here (cholesterol at a concentration of 7.6 pmol/ml was used). However, the different temperatures at which experiments were performed may result in a different critical micellar concentration. This reversible self association of cholesterol is believed to yield a rod shaped micelle with a molecular weight of approximately 200,000 Daltons. This would readily explain the elution of the cholesterol in the void volume.

When the experiments were repeated using propylene glycol as the solvent for the cholesterol as reported by Ritter and Dempsey (1973), the cholesterol was still found to be eluted in the void volume regardless of the gel used. This suggests that some aggregation of cholesterol also occurred in this solvent. In their report Ritter and Dempsey (1973) do not show the results obtained from a blank run, i.e. when the cholesterol alone was applied to the column, so one must presume that they did observe retention of the free cholesterol. It is therefore difficult to explain the different results observed. The presence of phospholipid has been reported to be necessary for the binding of cholesterol by high density lipoprotein (Sodhi and Gould, 1967). It is possible that the presence of phospholipid in the impure SCP preparation of Ritter and Dempsey (1973) may have influenced the results obtained. The results presented in this thesis, however, strongly support those of Scallen et al., (1985) who failed to show any involvement of FABP (i.e. SCP) in physiological reactions involving cholesterol transfer. They attribute cholesterol binding activity to another low molecular weight protein, SCP_2, which differs significantly from SCP in amino acid composition. They claim that the protein referred to in this thesis as SCP functions in fatty acid binding and/or transport. This study has confirmed the fatty acid binding of SCP; 1.2 pmoles oleic acid was bound per g SCP as it was isolated, i.e. without delipidation.

Maximum binding of dealbuminised rat liver cytosol was reported as being 4.39 pmol/μg protein by Glatz and Veerkamp (1983). This value is considerably higher than the values presented in this thesis for fatty acid binding by pure SCP. However, it should be noted that the fatty acid binding observed was reported to be dependent on the concentration of fatty acid used and that at low fatty acid concentrations, as were used in this study, prior delipidation of the test protein resulted in a significant increase in fatty acid binding. In the studies reported in this thesis SCP was not delipidated prior to faty acid binding studies. Finally, the effect of using cytosolic proteins, some of which may have fatty acid binding activity in addition to that exhibited by SCP, cannot be ruled out. The presence of such proteins could artifically elevate the apparent fatty acid binding activity of a single protein, in this case SCP.

This study was initiated following a report by Dempsey (1984a) that SCP was found in serum where it was primarily associated with the high density lipoprotein fraction. The mechanism of association of SCP with HDL was thought to be related to the cholesterol binding properties of SCP as was reported by Ritter and Dempsey (1971, 1973). However, in this study we were unable to demonstrate cholesterol binding by SCP which thus leaves the question of the mechanism of the reported association of SCP with HDL unanswered. However, it is possible that SCP may bind to the HDL particles via phospholipid mediated cholesterol binding. Therefore, further work incorporating phospholipid into the binding studies mentioned is required before an ability to bind cholesterol by SCP can be ruled out.

The third aim of this project was to carry out the initial stages in the development of a sensitive assay system for measurement of SCP levels in serum. It was believed that a sandwich enzyme linked immunosorbent assay would ultimately yield a suitable system. As mentioned earlier, such an assay would require two different antibody preparations with high specificity for SCP.

One antibody preparation, the solid phase antibody, would capture the antigen molecules from the sample. They would then be available for reaction with the second antibody preparation which would be enzyme-conjugated to permit detection. The two

antibodies need to be directed against different antigenic sites on the antigen in a sandwich assay unless the antigen is known to have some kind of symmetry and hence replicating antigenic determinants.

SCP is a relatively small protein (approximately 14,000 Daltons (by amino acid analysis, Dempsey, 1985)) and therefore some difficulty was expected in generating an immune response to this protein. Therefore, antibody production was approached from a number of different angles. Earlier studies indicated that SDS-PAG-incorporated SCP generated an immune response in rabbits (McGuire et al., 1984). Thus this was one of the selected methods for this study. This method has the advantage that the purity of the immunogen is ensured, being a single band from a previously run SDS-polyacrylamide gel. However, since SDS was required for better resolution of SCP from any possible contaminating proteins, the immunogen, SCP was not in its native state. Therefore, undenatured SCP was also used as immunogen in a second set of animals. There are various reports on the efficiency of various routes of immunization in rabbits. Hence both intradermal and subcutaneous sites were used with each immunogen. Following a report by Tung (1983), production of antibodies to both PAG-incorporated and native SCP in mice by intraperitoneal injection was also attempted.

During the course of this study only two antisera were produced with sufficiently high titre and exhibiting a high degree of selectivity and sensitivity. Both were produced following intradermal injection of native SCP into rabbits. Both were found to be able to detect SCP at levels of 0.2μg/well when SCP was coated directly onto the well and using a commercial second antibody-enzyme conjugate. The concentration of SCP in human serum has been reported to be in the range of 13.7 - 17.9 mg/dl (Cunningham, 1985). The average sample volume used in ELISA systems is 50 μl. This volume of serum would contain approximately 8 μg of SCP based on the above data. Thus, both antisera generated during this study are sufficiently sensitive for use in an assay of SCP in serum.

The specificities of both IgG preparations were found to be different. R_{284} only reacted with SCP while R_{289} reacted with

both SCP and BSA. The reaction with BSA was low but was, however, detectable under experimental conditions used. Cross-reaction of the antibody with BSA was not unexpected as both proteins have fatty acid binding properties and therefore, possibly have antigenic sites in common. The observed difference in specificity suggests that the two sets of antisera are directed against different antigenic determinants and therefore suitable for use in a sandwich assay for the reasons outlined earlier.

The assay system used in this study, involving direct binding of SCP to microtitre plate wells followed by reaction with one of the anti-SCP antibody preparations and use of a second antibody-enzyme conjugate for detection, is only suitable for measurement of purified SCP. The long-term aim of this work was to provide an alternative assay system for SCP in serum or serum fractions which would be simpler and less time-consuming than the immuno-precipitation assay used to date (Conneely et al., 1984).

It was not feasible during the present study to complete the development of the sandwich ELISA for SCP which is needed to measure SCP levels in complex mixtures such as serum. To achieve this goal an enzyme-anti-SCP conjugate would have to be prepared. A number of different methods are available for enzyme-antibody conjugate preparation. Much difficulty was encountered during this study with the stability of commercial second antibody-enzyme preparations. Both enzyme and immunological activities showed instability at various times. Details of methods of preparation were not available and so possible reasons for these problems were not clear. The HRP-anti rabbit IgG-conjugate finally used in this study, which was provided as a gift, was produced by Maleimide conjugation of the F ab portion of the antibody to HRP by the method of Ishikawa et al., (1983). This enzyme antibody conjugate preparation was found to be very stable when stored at $4^{o}C$. It is recommended that the anti-SCP-enzyme conjugate be prepared, initially, by a similar method and that due attention be given to checking stability of the product under different conditions of storage.

In conclusion, therefore, in this study SCP was isolated and purified from rat liver. The purified protein was used in conventional lipid binding studies which indicated that SCP is a fatty acid binding protein without the ability to bind cholesterol

under the experimental conditions examined. This work lends support to similar recent conclusions made about this protein based on experiments which investigated its physiological function. Two antisera of high specificity and sensitivity have been prepared and are available in sufficient quantities to be used in the further development of a sensitive and quantitative sandwich ELISA for SCP.

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6 APPENDIX

ABBREVIATIONS USED

SCP	Sterol Carrier Protein
SCP_1	Sterol Carrier $Protein_1$
SCP_2	Sterol Carrier $Protein_2$
SPF	Supernatant protein Factor
ns L-TP	Non-Specific Lipid-Transfer Protein
CRBP	Cellular Retinol Binding Protein
CRABP	Cellular Retinoic Acid Binding Protein
FABP	Fatty Acid Binding Protein
CSCC	Cholesterol Side Chain Cleavage
SDS	Sodium Dodecyl Sulphate
PAG	Polyacrylamide Gel
RIA	Radioimmuno Assays
EIA	Enzyme Immuno Assays
ELISA	Enzyme Linked Immuno Sorbent Assay
HRP	Horse Radish Peroxidase
Fab	F Antibody
HDL	High Density Lipoproteins
LDL	Low Density Lipoproteins
VLDL	Very Low Density Lipoproteins