AN INVESTIGATION OF THE ROLE OF CHOLESTERYL ESTER TRANSFER PROTEIN IN LIPID METABOLISM

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SUMMARY

The investigation of the role of cholesteryl ester transfer protein (CETP) in lipid metabolism was divided into five main areas. These included the development of a reliable and reproducible assay for CETP activity, the development of polyclonal and monoclonal antibodies to CETP, studying the effect of various drugs on plasma CETP activity in several animal species, looking at the control of CETP secretion by different cell lines and analysing the relationship between restriction fragment length polymorphisms (RFLPs) of the CETP gene and various plasma lipid parameters in a population of 56 individuals.

An assay was developed which measured an activity displaying the correct characteristics for CETP. The assay measured the accumulation of labelled lipid in an acceptor lipoprotein particle after transfer from a radiolabelled donor lipoprotein particle and separation of the donor and acceptor particles by heparin manganese precipitation. The separation of the lipoproteins by precipitation was validated using an independent lipoprotein separation method, ultracentrifugation. The assay could be adapted to measure low amounts of CETP activity by extending the length of incubation time.

Human and rabbit CETP were partially purified using previously published methods. This preparation was further purified by binding the CETP to Intralipid. For the production of monoclonal antibodies mice were immunised with both partially purified and further purified CETP. After fusion of mouse spleen cells with NS1 cells a clone was identified which produced antibodies which were positive in an ELISA using partially purified human CETP as an antigen and which could inhibit human CETP activity. This clone requires further development. Work on monoclonal antibodies to rabbit CETP was discontinued. An attempt to raise polyclonal antibodies to a synthetic peptide from the CETP sequence failed to produce antibodies which would recognise the native protein.

Plasma CETP activity was measured in rats in the middle of the light cycle and the middle of the dark cycle. This did not produce any evidence for a diurnal variation of plasma CETP activity in rats. Increased plasma CETP activity was demonstrated in fat-fed and genetically hyperlipidaemic Froxfield rabbits. The effect of drugs which affect lipid metabolism was studied in rabbits, marmosets and humans. No consistent effects of these drugs on plasma CETP activity were demonstrated.

Human monocyte derived macrophages, J774 cells, HepG2 cells and CaCo-2 cells were found to secrete very small and irreproducible amounts of CETP into the medium. Due to the difficulty of measuring such low levels of CETP activity an attempt was made to measure CETP messenger RNA levels in these cells. Due to technical difficulties quantitative measurement of messenger RNA was not achieved and it was not possible to assess how various effectors might control CETP secretion.

An StuI restriction fragment length polymorphism (RFLP) of the CETP gene was discovered. The relationship of this polymorphism, the TaqI polymorphism of the CETP gene and two apo AI polymorphisms to plasma total cholesterol, HDL cholesterol, CETP activity and LCAT activity was investigated in a population of 56 unrelated individuals

selected on the basis of their HDL levels. It was found that individuals expressing the rare TaqI B_2 allele were more likely to have high plasma HDL and low plasma CETP activity. This suggests that CETP may have an influence on plasma HDL levels.

CHAPTER I

INTRODUCTION

1 Discovery

Plasma lipoprotein cholesteryl esters (CE) were originally thought to be nonexchangeable. This concept was based on experiments from the 1960s. Roheim *et al* [Roheim, P.S. *et al* (1963)] demonstrated in the rat that there was no equilibration of cholesteryl esters between ^{14}C-cholesterol labelled chylomicrons and plasma *in vitro*; nor was there exchange of cholesteryl esters between high (d>1.063g/ml) and low (d<1.063g/ml) density lipoproteins, labelled biosynthetically, *in vitro*. Goodman [Goodman, D.S. (1964)] injected ^{14}C-mevalonate into two male volunteers to label their cholesterol moieties. He found that there were differences in the rate of appearance of radioactive cholesteryl ester in the plasma lipoproteins and that although labelled cholesterol did equilibrate between the lipoproteins, cholesteryl esters showed markedly different specific activities in the different lipoprotein fractions. Also the fractional turnover of cholesteryl ester differed between high density lipoprotein (HDL) and low density lipoprotein (LDL), being higher in the former and lower in the latter. The conclusion from these *in vitro* and *in vivo* experiments was that the pools of cholesteryl ester in different lipoprotein fractions were distinct and nonexchangeable.

Later evidence began to emerge which contradicted these early experiments. Transfer of CE and triglycerides (TG) between human lipoproteins *in vitro* was described by Nichols and Smith [Nichols, A.V. & Smith, L. (1965)]. After incubation of human serum for 36 hours at 38°C the isolated lipoprotein fractions were all found to have an increased cholesteryl ester content. It was proposed that this was due to synthesis of cholesteryl ester in HDL by lecithin cholesterol acyl transferase (LCAT) and subsequent transfer to other lipoprotein fractions [Rehnburg, C.S. & Nichols, A.V. (1964)]. HDL was also found to have increased triglycerides and it was suggested that these probably derived from the LDL fraction which showed a decrease in triglyceride.

In 1971 Quarfordt *et al* [Quarfordt, S.H., Boston, S. & Hilderman, H. (1971)] measured triglyceride mass transfer between very low density lipoprotein (VLDL)/LDL and VLDL/HDL donor/acceptor pairs at plasma concentrations in the absence of serum. No transfer was observed at 4°C; and at 37°C only transfer between HDL and VLDL was observed. Transfer was not affected by pH or ionic strength but was accelerated as

donor or acceptor concentration increased. The authors offered no mechanism for this transfer but it now seems likely that this was due to contaminating CETP (cholesteryl ester transfer protein) in the HDL fraction.

Evidence that the transfer reaction is important in man came from Nestel *et al* [Nestel, P.J., Reardon, M. & Billington, T. (1979)]. Following injection of labelled HDL into humans, VLDL cholesteryl esters were rapidly labelled, the apparent flux rate from HDL being similar to the rate of total cholesterol esterification in plasma. Barter and Lally [Barter, P.J. & Lally, J.I. (1978)] demonstrated that human plasma contained transfer activity whereas that from rats did not, regardless of the origin of the lipoprotein substrates. This difference between the species explained the contradictory results obtained by earlier investigators. Barter and Lally also calculated [Barter, P.J. & Lally, J.I. (1979)] that the rate of transfer was of the same order as the rate of cholesterol esterification in plasma and they argued that this dispelled the idea of discrete non-exchangeable pools of cholesteryl ester since CE could be transferred as quickly as they could be formed. If cholesteryl esters were nonexchangeable then it might be expected that the different specific activities for lipoproteins detected by Goodman [Goodman, D.S. (1964)] would have persisted for longer than the 48 hours observed since the half life of LDL and HDL is three or more days. Goodman assumed that since CE equilibration was not as rapid as that of the free sterol then no exchange was taking place. At the time the experiments were carried out it was not generally accepted that plasma CE are LCAT derived and that HDL was the primary substrate for LCAT. On the contrary it was thought that each lipoprotein could be synthesised independently from the others. Glomset reported in 1968 [Glomset, J.A. (1968)] that the initial rate of cholesterol esterification was similar to the turnover rate of CE. This idea is not consistent with lipoproteins having different turnover rates unless CE can be transferred.

An active factor which could stimulate the exchange of cholesteryl ester and triglyceride between VLDL and LDL was first identified in rabbit plasma [Zilversmit, D.B., Hughes, L.B. & Balmer, J. (1975)]. Since the total mass of cholesteryl esters in each lipoprotein did not change during this process it appeared that an exchange activity was being measured. The factor increased transfer in a concentration dependent manner and was found in the d>1.125g/ml fraction of plasma from both normal and hyper-cholesterolaemic rabbits. No transfer occurred at all without addition of this factor. The HDL fraction had a small stimulatory activity but the d>1.063 or d>1.21 g/ml fractions caused greatest transfer. It transpired that the catalyst was a high molecular weight plasma protein with isoelectric point 5.2. Transfer rates were the same in the presence and absence of an LCAT inhibitor (*p*-hydroxymercuribenzoate) and the factor was stable (1 hour at 56℃) under conditions where LCAT activity was destroyed. Therefore the active factor was unlikely to be LCAT.

2 Purification

The first satisfactory purification of a protein catalysing the exchange of cholesteryl esters was carried out by Pattnaik *et al* [Pattnaik, N.M. *et al* (1978)]. A glycoprotein,

distinct from any of the known apolipoproteins was purified 3500 fold with apparent molecular weight 80000. At the same time Fielding's group [Chajek, T. & Fielding, C.J. (1978)] isolated a human serum CETP, with molecular weight 35000, from the HDL fraction and showed it to be immunologically unreactive to antibodies to the major HDL apolipoproteins AI, AII and E. Antibodies were raised to this protein and could remove transfer activity from HDL and d>1.21g/ml plasma. Since the purification procedure used was similar to that for apolipoprotein D (apoD), the authors suggested that the transfer protein and apoD may be identical. A triglyceride transfer protein was also isolated from rabbit plasma [Rajaram, O.V., White, G.H. & Barter, P.J. (a)(1980)]. Several groups followed suit most employing the basic steps of hydrophobic and ion exchange chromatography with variations thereafter. In general molecular weights of 58-66K and pIs of 5.0 - 5.2 were quoted [Morton, R.E. & Zilversmit, D.B. (1982), Albers, J.J. *et al* (1984), Bastiras, S. & Calvert, G.D. (1986)] though one group [Ihm, J. *et al* (1980)] reported a much greater molecular weight of 150K. The protein appeared to catalyse both the exchange and mass transfer of lipid; investigators finding one or both activities in their studies. Sometimes purified activity appeared to run as a doublet of about 58 and 63K [Ihm, J. *et al* (a)(1982), Morton, R.E. & Zilversmit, D.B. (1982), Albers, J.J. *et al* (1984), Bastiras, S. & Calvert, G.D. (1986)] but each band showed equivalent transfer activity. These bands were thought to be the same protein in different forms or glycosylated to different extents.

The transfer protein was initially identified by immunoreactivity as apoD [Fielding. P.E. & Fielding, C. (1980), Chajek, T. & Fielding, C.J. (1978)]. This idea was disputed [Morton, R.E. & Zilversmit, D.B. (a)(1981)] when apo D and CETP were separated by gel filtration and electrophoresis and was finally dispelled when the apoD and CETP genes were cloned [Drayna, D. *et al* 1986, Drayna, D. *et al* (1987)].

Later investigators purified to homogeneity transfer proteins which appeared to have the same molecular weights and properties. In 1987 Hesler *et al* [Hesler, C.B., Swenson, T.L. & Tall, A.R. (1987)] purified the transfer protein 55000 fold using its affinity to bind a lipid emulsion. This preparation had a molecular weight of 74K. The protein had a high hydrophobicity, calculated to be greater than any other plasma apolipoprotein. Antibodies to this protein completely removed activity from partially purified fractions. It was proposed that the doublet observed with polyacrylamide gel electrophoresis (PAGE) represented isoforms of the protein. The N terminal amino acid composition was different from the protein purified by Ihm *et al* [Ihm, J. *et al* (1982)] and was also different from apoD. This protein could be easily degraded by the presence of lipid peroxides and degradation did lead to the production of lower molecular weight forms but only in the presence of lipid peroxide containing emulsions. The lower molecular weight forms seen could be related to the protein by degradation. Jarnagin *et al* [Jarnagin, A.S., Kohr, W. & Fielding, C. (1987)] also isolated a 74 kDa protein, purified 100000 fold, which had an isoelectric point of 5.2. They found that the previous doublet [Ihm, J. *et al* (1982)] was due to contamination and bands could be separated by isoelectric focussing. The 74K protein was in fact the one responsible for transfer activity.

The molecular weight of rabbit CETP was determined by radiation inactivation, a technique not requiring purified enzyme and not influenced by shape. It was found to be 70000 +/- 3000 and suggested that the protein was a monomer [Ierides, M. *et al* (1985)]. The human cholesteryl ester transfer protein cDNA was cloned and sequenced in 1987 [Drayna, D. *et al* (1987)]. From the deduced amino acid sequence the calculated translated molecular weight was 53K. The high molecular weight detected previously may be accounted for by glycosylation and four potential asparagine linked glycosylation sites do occur in the molecule. Discrepancies in molecular weights between groups may also be accounted for by degradation effects or impurities.

Evidence that these preparations catalysed both cholesteryl ester and triglyceride exchange raised the question as to whether these activities proceeded under the influence of one protein, either on a single non-specific site or on different sites, or several proteins in a complex. A single transfer protein activity is suggested by several lines of evidence. Firstly the species distribution of cholesteryl ester and triglyceride transfer activities reflect similar expression of these activities. It was found that cholesteryl ester and triglyceride transfer activities co-purified through all purification stages. Secondly the two enzyme activities also show identical inactivation profiles by denaturation either by heat or by proteolytic enzymes [Morton R.E. & Zilversmit D.B., (b) (1981)]. Thirdly the kinetics of the interaction with substrates were similar for both the triglyceride and the cholesteryl ester transfer activities [Rajaram, O.V. & Barter, P.J. (1980)]. Finally variation of LDL and HDL concentration produces similar effects on both cholesteryl ester and triglyceride transfer activity. However triglyceride transfer was inhibited by mercurial compounds e.g. parachloromercuriphenylsulfonate whereas cholesteryl ester transfer was not [Morton, R.E. & Zilversmit, D.B. (1982)]. It has been concluded from this evidence that cholesteryl ester and triglyceride transfer are carried out by the same protein [Morton, R.E. & Zilversmit, D.B. (1982), Albers, J.J. *et al* (1984)] and that differential inhibition by mercurial compounds could be explained by these transfer activities being carried out at different sites on the same molecule. Monoclonal antibodies to the purified 74K CETP caused parallel and complete inhibition of cholesteryl ester and triglyceride transfer activities but only partial inhibition of phosphatidyl choline (PC) transfer *in vitro* [Hesler, C.B. *et al* (1988), Abbey, M. & Calvert, G.D. (1989)]. Thus the 74K protein accounts for all TG and CE transfer activity in human plasma.

3 Other Transfer Proteins

An intracellular CE and TG transfer protein has been identified in bovine liver. It has an apparent molecular weight of 200K and isoelectric point between 5.2 and 5.6. This may be similar or identical to the plasma transfer protein. However, this protein showed a preference for transferring TG over CE [Wetterau, J.R. & Zilversmit, D.B. (1984)]. A similar activity has been isolated from rat liver [Wetterau, J.R. & Zilversmit, D.B. (1986)] and appears to be associated with the microsomal fraction. Similar activities

were identified in intestinal mucosa but not in brain, heart, kidney or plasma. This protein is possibly involved in the incorporation of CE and TG into precursors for lipoprotein synthesis by the liver and intestine.

Although both CE and TG transfer activities in plasma could be accounted for by one protein, PC transfer seemed to be carried out by at least two. Albers *et al* [Albers, J.J. *et al* (1984)] separated PC transfer activities on heparin Sepharose columns. The unbound fraction was temperature resistant and eluted with temperature resistant CE and TG transfer activities. This fraction was called LTP-I. The second activity bound to heparin sepharose, was temperature sensitive and facilitated transfer of PC but not CE or TG. This activity was designated LTP-II. It seems that LTP-I is identical to CETP. A PC transfer protein could also be separated from a CE/TG exchange protein by ion exchange chromatography [Tall, A.R., Abreu, E. & Shuman, J. (1983)]. The protein, which catalyses phospholipid (PL) transfer only was later purified [Tollefson, J.H., Ravnik, S. & Albers, J.J. (1988)]. It catalyses exchange and net mass transfer of PL, does not transfer CE or TG, is not recognised by antibodies to LTP-I and has an isoelectric point of 5.0. The molecular weight for this activity has been reported as 70K [Tollefson, J.H. *et al* (1988)], 41K [Tall, A.R. *et al* (1983)] and 65K [Albers, J.J. *et al* (1984)]. LTP-II enhances transfer of CE from HDL_3 to VLDL when incubated with LTP-I. Two roles are proposed for net mass transfer of PC [Albers, J.J. *et al* (1984)]. First to remove free surface coat materials after lipolysis of TG rich lipoproteins and secondly to provide substrate for the LCAT reaction. Consideration of these phenomena (Table 1.1) suggests that the transfer activity in the rat may be explained by the animal expressing LTP-II but not LTP-I.

4 Localisation of CETP on Lipoproteins

CETP is thought to be located in the HDL and d>1.215g/ml fractions of plasma [Groener, J.E.M., Van Rozon, A.J. & Erkelens, D.W. (1984)]. Activity eluted with HDL when plasma was passed down a gel filtration column but eluted in the position for free CETP when d>1.215g/ml fraction was used [Pattnaik, N.M. & Zilversmit, D.B. (1979), Rajaram, O.V. & Barter, P.J. (1980)]. Groener *et al* [Groener, J.E.M. *et al* (1984)] located CETP mainly on HDL_3 and Cheung *et al* [Cheung, M.C. *et al* (1986)] reported CETP to reside mainly on HDL particles containing apo AI and no apo AII. The activity could be separated from these particles by ultracentrifugation. CETP activity separated from the HDL fraction during centrifugation is found mainly in the d>1.215g/ml fraction. About 80% of HDL-CETP complexes are disrupted by single step ultracentrifugation.

CETP was found to form a complex with HDL but not LDL or VLDL after passage through a gel filtration column [Pattnaik, N.M. & Zilversmit, D.B. (1979)]. This interaction was thought to be electrostatic possibly due to interactions with the phosphate head groups of the phospholipids. Morton [Morton, R.E. (1985)] looked at its interaction with Sepharose bound lipoproteins (LP). HDL formed stable complexes with CETP but VLDL/LDL-CETP complexes were more labile. CETP bound to all lipoproteins showing high affinity saturation binding under equilibrium conditions. The Kds were about 25nM.

TABLE 1. PROPERTIES OF CHOLESTERYL ESTER TRANSFER PROTEIN AND PHOSPHATIDYL CHOLINE TRANSFER PROTEIN

PROPERTY	CETP (LTP-I)	PC TRANSFER PROTEIN (LTP-II)
Molecular Weight:		70000,41000,65000
apparent	74000	-
calculated	56000	-
Isoelectric point	5.2	5.0
CE/TG exchange	+	-
PC exchange	+	+
Heparin Sepharose Binding	-	+
Temperature Resistance	+	-
Precipitation by anti LTP-I	+	-

CETP binding and transfer increased in parallel with increasing CETP concentration. Inhibiting CETP caused decreased CETP binding to lipoproteins. It is possible that inhibition disrupts CETP-LP complexes. It was concluded that CETP avidly binds VLDL/LDL and HDL via a reversible saturable mechanism and that binding of CETP to lipoproteins is an integral part of the transfer reaction. Binding studies showed that VLDL,LDL and HDL have approximately the same affinity for CETP but markedly different binding capacities at saturating levels for CETP. The authors estimated that the minimum binding capacity of lipoproteins for CETP exceeds the plasma concentration of CETP by at least 10 fold. HDL alone has a binding capacity greater than the plasma CETP concentration and could therefore essentially bind all CETP dissociated from LDL and VLDL during gel filtration. Thus it appears that lipoproteins in physiological concentrations are not saturated with CETP. Changes in HDL and LDL/VLDL pool size may be accompanied by shifts in CETP distribution which may explain in part the abnormal CETP activity found in patients with abnormal lipoprotein profiles. CETP and LCAT can associate with HDL_2 and HDL_3; [Nishida, H.I., Kato, H. & Nishida, T. (1988)] and apoAI decreases their association.

The transfer protein may exist in a complex along with other proteins involved in cholesterol transport. Although purified CETP elutes at about 68K on gel filtration, if whole plasma is chromatographed the transfer protein elutes with particles of much larger size (160-320K). This suggests that the CETP may exist in a complex [Tollefson, J.H. & Albers, J.J. (1986)]. Large molecular weight complexes have been isolated by gel filtration chromatography [Ihm, J. *et al* (1980)] and by immunoadsorption chromatography [Chajek, T. & Fielding, C.J. (1978), Fielding, P.E. & Fielding, C.J. (1980)]. LCAT, transfer activity and apoAI could be isolated as a complex. When plasma was briefly incubated with ^{3}H-cholesterol loaded fibroblasts labelled CE first appears in an apoAI containing species (named LpAI pre β_3) which also contains apoD, LCAT and CETP [Francome *et al* (1989)].

Busch *et al* [Busch, S.J. *et al* (1987)] reported that the transfer protein existed as a high molecular weight fraction, greater than 150K, and low molecular weight fractions of 18K when the transfer activity was purified by immunoaffinity chromatography and then gel filtered on an HPLC system. Cholesteryl ester and triglyceride transfer specific activities were higher in the lower molecular weight fraction. They identified a low molecular weight proteolipid consisting of a catalytic domain (3K) and a heparin binding domain which could be differentiated from each other by antibody binding. This work has not been confirmed by other groups.

Hesler *et al* [Hesler, C.B. *et al* (1989)] looked for an active domain fragment of CETP by protease digestion or expression of CETP cDNA restriction fragments in Escherichia coli. Protease treatment of CETP with trypsin or chymotrypsin did not result in loss of catalytic activity although the protein was degraded. Incubation with Staphylococcus aureus V8 proteinase resulted in partial loss of activity. Despite substantial proteolysis by three different proteases the monoclonal antibody to the 75K purified transfer protein, TP2, was still able to inhibit transfer activity. Thus the active structure

contains an intact epitope for this antibody. Gel filtration of the products of proteolysis showed that they eluted in the same place as the whole protein indicating that after degradation the polypeptide fragments remain associated and retain the native molecular weight of CETP. These complexes were very stable and resistant to dissociation with urea, dithiothreitol or delipidating agents. CETP has a distinct and highly stable tertiary structure required for activity. The fragments which were expressed in E. coli were catalytically inactive though they did still contain an epitope for a monoclonal antibody which can inhibit CETP activity; and they still have similar emulsion binding properties to the whole protein. Therefore there is no evidence for an independently active subunit of CETP contrary to other reports [Busch, S.J. *et al* (1987)].

5 Mechanism

Investigations were carried out into the substrate specificity of CETP using reconstituted HDL and liposomes as donors [Morton, R.E. (1986)]. The relative transfer rate of different cholesteryl esters was cholesteryl oleate > cholesteryl linoleate > cholesteryl arachidonate > cholesteryl palmitate. Transfer with cholesteryl oleate was about 154% that of cholesteryl palmitate. All major species of ester were transferred and the relative rates are due to differences in the acyl chain. This reflects substrate specificity rather than availability of the substrates in the lipoprotein particle for transfer. Differences in substrate specificity would lead to a different equilibration of cholesteryl esters between lipoproteins and may result in altered metabolism of lipoproteins. Since the composition of CE in the outer layer affects the fluidity of the lipoprotein particle, reactions such as VLDL to LDL conversions and lipoprotein remodelling may be affected.

Equimolar back transport of TG has been demonstrated in incubations of ^{14}C-CE labelled HDL and ^{3}H-TG labelled LDL or VLDL with plasma thus demonstrating a coupled exchange of HDL cholesteryl ester for lower density lipoprotein triglyceride [Chajek, T. & Fielding, C.J. (1978)]. Jarnagin *et al* [Jarnagin, A.S. *et al* (1987)] found that the CE transfer rate was greater than that for TGs between each of the major lipoprotein classes using purified CETP. They suggested that CETP has a preference for CE and equimolar transport is the result of the combined activity of different transfer activities in plasma. Morton and Zilversmit [Morton, R.E. & Zilversmit, D.B. (1983)] looked at CE,TG and PC transfer. Looking at initial transfer rates (i.e. less than 10 - 13% transfer), absolute transfer rates were affected by the donor and acceptor LP concentration and acceptor LP composition, whereas the ratio of CE:TG transfer was not. This was also found to be the case using unilamellar vesicles as substrates. No correlation was found between the amount of PL transferred and the amount of CE/TG transferred. The authors concluded that TG and CE compete for transfer and that the extent of transfer depends on the relative concentrations of these lipids in the donor particle. PL transfer is independent. These data were from exchange reactions not net transfer, and experiments were carried out using pure CETP i.e. in the absence of LCAT. Using liposome acceptors it was found that inhibition of TG transfer with para chloromercuriphenylsulphonate (pCMPS) also suppresses CE transfer from LDL. It seems

that net transfer of CE and TG is coupled such that one lipid is transferred in one direction and the other in the opposite direction. Exchange versus net transfer (heteroexchange) may be dependent on core composition of donors and acceptors. Human LDL and HDL have nearly identical CE to TG ratios whereas the ratios in LDL and HDL differ greatly in rat. Transfer to the TG rich VLDL particles found in liver perfusate was similar to transfer to plasma VLDL (which is more cholesteryl ester rich). This implies that in normal rabbit plasma the lipid composition of acceptor lipoproteins is not rate limiting for transfer [de Parscau, L. & Fielding, P.E. (1984)].

Several studies were carried out to investigate the mechanism of the transfer reaction. Rates of exchange depended on both LDL and HDL concentration [Ihm, J. *et al* (b) (1982)]. If the ratio of LDL:HDL concentrations exceeded 9:1 then flux rates were independent of LDL at constant HDL concentration. Exchange was inhibited at high HDL concentrations when LDL concentration was constant, which the authors argued demonstrated preferential HDL/HDL exchange but may have been due to the presence of inhibitor. Analysis of the dependence of initial exchange on LDL concentration at two constant HDL concentrations suggested a productive collision i.e. ternary complex, mechanism. At low HDL concentrations this complex is formed by a random sequence route. At high HDL concentration the mechanism may be ordered sequential since CETP and HDL will already exist as a complex. These studies looked at exchange only. They suggest that the free energy of the complex would be reduced if CETP entered the complex already bound to a lipoprotein. The reaction would be second rather than third order. Sammett and Tall [Sammett, D. & Tall, A.R. (1985)] showed that increased CETP activity is associated with an increased binding of CETP to VLDL remnants and HDL, which is consistent with Ihm's model.

Barter *et al* fitted transfer data to a mathematical model [Barter, P.J. *et al* (1982)]. The model assumed that the transfer protein interacts with the lipoprotein particles and may bind. If there are equal concentrations of cholesteryl ester in each lipoprotein fraction then the probability of picking up cholesteryl ester in HDL:VLDL:LDL is 28.0:4.65:1. This predicts that at physiological lipoprotein concentrations the proportion of transfer protein bound to HDL is more than double that unbound. For VLDL the prediction is that one tenth is bound. These predictions however apply to a closed system not to a dynamic one as would be present *in vivo*.

Swenson *et al* [Swenson, T.L., Brocia, R.W. & Tall, A.R. (1988)] studied the mechanism of the CETP reaction using binding to unilamellar vesicles and then gel filtration chromatography. They looked at transfer of radioactive CE/TG and PC to CETP and then isolated the complexes. The isolated CETP labelled lipid complex can donate to LDL. Parachloromercuriphenylsulfonate, which inhibits CE and TG transfer decreases the binding of radiolabelled CE and TG to CETP. These experiments show binding sites for CE and TG on CETP which can equilibrate readily with lipoprotein lipids. This suggests that CETP can act as a carrier between lipoproteins. These findings fulfil two criteria of a carrier mediated mechanism. One is the isolation of a carrier bound with lipid and second is the ability of the carrier complex to donate bound lipid

to a lipoprotein. Apo-AI, apoHDL and albumin do not demonstrate these properties. Scatchard analyses of these data demonstrate a single CE and TG binding site. Bound phospholipid appears to play a role since extensive delipidation of CETP leads to inactivity which can be retrieved by the addition of PC by PC/cholate dialysis. PL may form part of the hydrophobic lipid binding site.

6 Lipid Transfer Inhibitor Protein

Species differences in transfer activity were observed and led to the initial confusion as to whether plasma lipoprotein CE were exchangeable. Early investigators, studying the rat, were led to believe that there was no equilibration of cholesteryl ester between lipoproteins. It was only in later studies when humans and rabbits were investigated that the transfer of triglyceride and cholesteryl ester was seen.

Rat serum showed much less (or negligible) transfer activity than human serum regardless ofthe species origin of the lipoprotein substrates [Barter, P.J. & Lally, J.I. (1978)]. Rabbit serum was much more effective than human. Guinea pig serum showed activity intermediate between that of human and rat [Barter, P.J. & Lally, J.I. (1979)]. A comprehensive study of cholesteryl ester transfer activity in lipoprotein free plasma of 16 species was carried out [Ha, Y-C. & Barter, P.J. (1982)]. It was found that transfer activities could be divided into high, intermediate and low activity groups. Rabbits were high, humans intermediate and rats low.

It is now thought that the reason for this species variation is the presence of an inhibitor of CETP expressed to varying extents in the plasma of different species. It was found that rat and pig lipoprotein depleted plasmas (LPDPs) inhibited rabbit transfer activity [Zilversmit, D.B. *et al* (1975)]. An inhibitor of CETP could be separated from CETP by phenyl Sepharose chromatography. It was characterised as a protein of molecular weight 35000 and isoelectric point 4 or less. CETP activity was inhibited between all pairs of low, very low and high density lipoproteins [Morton, R.E. & Zilversmit, D.B. (b) (1981)]. Further work on inhibition showed that apoAI also inhibited CETP activity (though triglyceride transfer was inhibited more than cholesteryl ester transfer). Inhibition was independent of CETP concentration but was decreased by higher lipoprotein concentrations. Inhibition was not observed between liposomes. All these points taken together indicated that the inhibitor interacts with substrates rather than with the transfer protein [Son, Y-C. & Zilversmit, D.B. (1984)]. The inhibitor may work by interfering with the interaction between CETP and its substrates.

A CETP inhibitor which could also inhibit LCAT and LTP-II was reported. Chromatography of LPDS over phenyl Sepharose resulted in enhanced transfer activity for human serum and unmasking of activity in rat and pig plasma [Tollefson, J.H., Liu, A. & Albers, J.J. (1988)]. Using pig plasma, an inhibitory activity could be eluted from this column with ethanol. Two to three times more inhibitory activity could be eluted than was required to inhibit the pig transfer activity obtained from the initial phenyl

Sepharose eluate. This inhibitory component was found to be part of HDL. ApoHDL still inhibited CETP and whole HDL added to a transfer assay caused inhibition. Only the HDL(AI with AII) fraction inhibited and not the HDL(AI without AII) fraction.

Lipid transfer inhibitor protein (LTIP) was isolated and characterised in 1989 [Nishide, T., Tollefson, J.H. & Albers, J.J. (1989)]. It has an apparent molecular weight of 28000 and an approximate isoelectric point of 4.6. A particular subclass of HDL with a particle size smaller than that of whole HDL and which only contained a small amount of apoAI was a rich source of LTIP. About 85% of the inhibitory activity was found in this HDL fraction. LTIP inhibited cholesteryl ester, triglyceride and phosphatidyl choline transfer of CETP (LTP-I) and to a lesser extent PC transfer by the PC transfer protein, LTP-II. Antibodies developed to LTIP could unmask CETP activity when human, rat or pig plasma were passed down an immunoaffinity column. Increases in activity were 17%, 125% and 200% respectively. LTIP was found to be distinct from apoAI and it is possible that the inhibitory activity previously shown by apoAI is due to contaminating LTIP.

7 Source of CETP

Liver, which is the source of LCAT, was tested to see whether it was also a source of CETP. Two groups [Abbey, M. *et al* (1984), de Parscau, L. & Fielding, P.E. (1984)] detected CETP in rabbit liver perfusate alongside LCAT secretion. The first group measured activity after concentration of the perfusate and separation by hydrophobic chromatography and the second measured a linear increase of activity using reperfusion. Parscau and Fielding found that the accumulation of CETP and LCAT in the perfusate could be blocked by colchicine which inhibits secretion at the post Golgi stage and may involve disruption of microtubules. This indicates that these proteins are liver secretory products. Both exchange and mass transfer activity were demonstrated for the secreted transfer protein, exchange being about twice that of mass transfer. Transfer was more rapid to VLDL than to LDL i.e. the half-maximal rate of transfer to VLDL was higher than that to LDL.

CETP was also shown to be secreted in culture by human monocyte derived macrophages and two murine macrophage-like cell lines, J774 and P338D [Tollefson, J.H. *et al* (1985)]. The transfer protein secreted had similar properties to those already described i.e. molecular weight 62K, isoelectric point 5.0, binding to phenyl sepharose but not to heparin sepharose, transfer of CE, TG and PL and stability to heating at 56℃ for one hour. This secretion could be completely inhibited by 10μM cycloheximide or enhanced about 67% by 16μM phorbol myristate acetate (PMA).

HepG2 (and Hep3B) cells were also found to secrete CETP [Faust, R.A. & Albers, J.J. (1987)]. If human hepatocytes secrete CETP at a similar rate *in vivo* then they could account for all the CETP in plasma. This secretion could be completely inhibited by 5μM cycloheximide. The transfer protein secreted had similar properties to plasma CETP including immunoinhibition by anti-LTP-I antibodies. Secretion tailed off after 24 hours. This appeared not to be due to instability or down regulation of synthesis since the same

phenomenon occurred when samples were collected serially from the same dish i.e. the cumulative activity from cells incubated with fresh medium each time. Therefore it is probably due to lack of serum factors required for synthesis. After passage down a heparin Sepharose column transfer activity increased. This suggests that HepG2 cells also secrete an inhibitor. This inhibitor could inhibit either HepG2 cell secreted or plasma-derived CETP. Apart from its ability to bind heparin Sepharose and to inhibit both cell secreted and plasma-derived CETP there is no evidence to positively identify it as LTIP.

The secretion of CETP by HepG2 cells was confirmed by Swenson *et al* [Swenson, T.L. *et al* (1987)]. The secreted CETP could be removed from the media by precipitation with antibodies to plasma CETP and had a similar molecular weight (72-76K) to plasma CETP. The precipitated band was broad and diffuse on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) suggesting that the protein was a glycoprotein. Treatment of the secreted protein with neuraminidase or glycopeptidase F caused the protein to show increased mobility and a sharper band on SDS PAGE with a molecular weight of 58000. CETP precipitated from a cellular homogenate had a molecular weight of 58000. This suggests that the peptide is processed to give an N-linked carbohydrate. It is also known that CETP cDNA codes for a 53000 protein [Drayna, D. *et al* (1987)]. Tunicamycin, which inhibits N-linked glycosylation, reduced CETP secretion by 90% and no smaller forms were immunoprecipitated. Removal of carbohydrate from active CETP did not greatly effect its activity.

CaCo2 cells secrete CETP vectorially [Faust, R.A. & Albers, J.J. (1988)] into the lower, baso-lateral, chamber of Transwells, a cell culture system whereby cells are grown on a membrane and both surfaces of the cell are exposed to culture medium. Over 24 hours they secrete CETP at twice the rate of HepG2 cells. This secretion is regulated by fatty acids. It was speculated that the intestine may be the principal source of CETP and its synthesis is upregulated during the acute lipaemia which follows digestion. Chronic lipaemia may also affect its release.

Faust, Cheung and Albers [Faust, R.A., Cheung, M.C. & Albers, J.J. (1989)] looked at the distribution of secreted CETP with nascent lipoprotein particles in HepG2s. Sixty percent was associated with apoAI containing particles similar to the distribution shown in human plasma. However a large amount was also found associated with apoAII containing particles. This suggests that CETP distribution is modified by a post secretory process. No CETP was found associated with apoB. Mixing of cell media with anti-apoE matrix resulted in an increase in CETP activity. It is possible that there is an inhibitor associated with apoE containing particles. The purified inhibitory activity from heparin Sepharose had similar thermostability properties to LTIP.

8 The CETP Gene

The human CETP cDNA was cloned and sequenced in 1987 [Drayna, D. *et al* (1987)]. Nucleotide probes were made using the amino terminal amino acid sequence of the purified 74K protein. These probes were used to pull out sequences from a human

cDNA library. The translated sequences closely matched the amino acid sequence available. Sequences were eventually found which coded for part of the secretion signal prepeptide and 476 amino acids of the mature protein. The sequence of the mature protein coded for a protein of molecular weight 53108 with four potential glycosylation sites. Sequence analysis showed the protein to be more hydrophobic than any of the known apolipoproteins and lipoprotein metabolising enzymes. The C terminus has the most hydrophobic stretch which may be consistent with a possible neutral lipid binding site. Northern blots detected a CETP mRNA of 1900 +/- 50 nucleotides. Messenger RNA synthesis was detected in human liver, small intestine and adrenal gland to equal extents. Spleen also demonstrated a lot of message but this may be due to the macrophages residing there since macrophages are known to secrete CETP [Tollefson, J.H. *et al* (1985)]. Little or no message was detected in HepG2 cells, U937 cells, pancreas, leukocytes or placenta but CETP mRNA can be detected in isolated hepatocytes. Translation of the full length CETP cDNA in a eukaryotic system (Cos cells), but not a prokaryotic system (E coli.) [Hesler, C.B. *et al* (1989)] produced an active CETP. It is probable that post translational processing is required for expression of activity.

Two RFLPs for the TaqI restriction enzyme were found at the human CETP locus using the 1581 base pair clone as a probe [Drayna, D. & Lawn, R. (1987)]. An uncharacterised MspI polymorphism was also found. The chromosomal location of the CETP gene was identified as 16q21 [Sparkes, R.S. *et al* (1987)].

The human sequence was used to pull out the rabbit cDNA from a liver cDNA library [Nagashima, M., McLean, J.W. & Lawn, R.M. (1988)]. The amino acid sequence was determined from the sequences of cloned DNA and genomic DNA. The rabbit amino acid sequence was longer than that in human, 496 amino acids compared with 476 amino acids and was 81% homologous. The predicted molcular weight is 54400 and there are six potential glycosylation sites. The mRNA was 2.2kB and had an unusual polyadenylation sequence. CETP mRNA was mainly found in rabbit liver with smaller amounts in the adrenals and kidney. In contrast to the human none was found in the spleen though this may be due to differences in macrophage content. The human and rabbit probes were used to detect CETP message in different species. Message was detected in the human, rhesus monkey and rabbit but none was found in pig, mouse and rat. This distribution agrees with plasma levels of CETP, though plasma levels may only reflect expression of inhibitor and not expression of CETP. It is difficult to know to what extent this result is influenced by probe cross reactivity.

9 Factors Affecting CETP Activity

Factors affecting CETP activity apart from amount of CETP, amount of inhibitor, lipoprotein binding and substrate composition include apolipoproteins, enzymes and various pharmacological agents. Nishikawa *et al* [Nishikawa, O. *et al* (1988)] demonstrated that apolipoproteins were required to stabilise micellar structures to allow transfer activity to take place. Apo D can enhance CETP activity between an artificial donor and an LDL acceptor [Nishida, H.I., Tu, A. & Nishida, T. (1988)]. CETP will transfer between

HDL and Lp(a) but only at about half the normal rate. If the apo(a) is removed then the rate returns to normal. This indicates that transfer may depend on surface area [Groener, J.E.M. & Kostner, G.M. (1987)]. Lp(a) blocks the surface availability for transfer.

Lipolysis has been shown to enhance transfer between HDL and apoB containing lipoproteins [Castro, G. & Fielding, C.J. (1985)]. When post prandial VLDL and LDL were incubated with fasting plasma, transfer increased. It therefore seems that the properties of the lipoproteins are changed in fed plasma. Studies of transfer of CE from HDL to VLDL *in vitro* revealed that lipoprotein lipase enhances CETP activity [Sammett, D. & Tall, A.R. (1985)]. This stimulation of activity was correlated with the accumulation of fatty acids in VLDL remnants. If this accumulation is decreased, by incubation with fatty acid free albumin, then a parallel decrease of the stimulation of CE transfer occurs. If sodium oleate is added to VLDL with albumin, then stimulation of CETP activity is observed. If the pH is lowered from 7.5 to 6.0 the stimulatory effect is abolished probably due to protonation of the free fatty acids. Phospholipase A2 and a bacterial lipase that lacked phospholipase activity also stimulated CETP activity. If VLDL and HDL are lipolysed and then isolated and tested in a CETP assay both substrates stimulate CETP activity binding more CETP, though there is greater binding to VLDL than to HDL. CETP also binds lipolysed PL/TG emulsions. Those conditions which decrease stimulation of transfer also decrease CETP binding to remnants, emulsions and HDL. Stimulation is related to the accumulation of fatty acids in lipoproteins . This accumulation may augment lipoprotein binding and increase the efficiency of transfer. Lipolysis may increase CETP binding to VLDL and HDL and facilitate the formation of a collision complex. Also the relative increase in VLDL binding may oppose HDL to HDL exchanges.

When CETP activities were compared in fasted and postprandial plasma it was found that transfer was two to three times greater in the post prandial condition [Tall, A., Sammett, D. & Granot, E. (1986)]. Agarose chromatography of fasting plasma showed that most CETP was associated with small HDL particles and some CETP was free. Post prandial plasma showed increased binding of CETP to large HDL and an increased amount of CETP associated with apoB containing lipoproteins. Total CETP activity was 1.1-1.7 times higher in post prandial or delipidated post prandial plasma when assayed with exogenous lipoproteins. There was increased binding of purified CETP to HDL in post prandial compared with fasting plasma but total activity was unchanged. Thus increased activity in post prandial plasma may be due to any of these factors; increased TG rich acceptor lipoproteins, lipolysis enhanced binding of CETP to lipoproteins (especially large HDL), and increased CETP mass.

Prostaglandin E_1 at concentrations as low as 10^{-10}M, stimulates the transfer of PC and CE from HDL_3 to LDL [Muzya, G.I. *et al* (1987)]. This effect is seen with spontaneous transfer as well as facilitated transfer. PGE_2 and $PGF_2\alpha$ had no effect. LCAT activity is also enhanced probably due to PGE_1 induced reorganisation of the HDL surface.

Ritter *et al* [Ritter, M.C., Bagdade, J.D. & Subbaiah, P.V. (1989)] studied the effects of treating hypercholesterolaemic subjects with marine lipids , i.e. omega-3 fatty acids for three months. Plasma total cholesterol, free cholesterol, VLDL cholesterol and LDL cholesterol all decrease. Plasma triglycerides and HDL cholesterol remain unchanged. Before treatment all the patients had accelerated cholesteryl ester transfer activity (CETA). Treatment decreased this. Experiments combining lipoproteins from treated and untreated plasma showed that the acceleration of CETA in untreated patients was due to an enhanced avidity of VLDL for CE.

When FH patients are given probucol then their xanthomas regress [Matsuzawa, Y. *et al* (1988)]. This regression correlates with the decrease in HDL cholesterol. The HDL fraction in these patients were studied and it was found that the treated patients had smaller HDL_2. Probucol was given to two familial hyperalphalipoproteinaemic (FHALP) patients one who had impaired, but not deficient CETA, and one with normal CETA. Both showed net transfer of cholesterol from LDL/VLDL to HDL (measured in whole plasma with LCAT inhibited) rather than from HDL to LDL/VLDL as shown in seven normal samples. Treatment normalised the net transport of CE and decreased HDL_2 cholesterol. The authors suggested that probucol may increase reverse cholesterol transport. This finding was confirmed [Sirtori, C.R. *et al* (1988)] when it was found that treating type II hyperlipidaemic patients with probucol resulted in an increase transfer rate from HDL to lower density lipoproteins. The HDL_2 fraction was decreased while HDL_3 was unchanged. It was suggested that probucol enhances HDL particle inter-conversion.

Since hepatic cholesterol secretion is increased in hypercholesterolaemics as is CETA, it is possible that CETP activity is regulated by hepatic cholesterol secretion. Rabbits were fed a coconut oil diet which lead to a 68% increase in plasma cholesterol, a 42% increase in CETA and a 69% increase in hepatic cholesterol secretion [Quig, D.W. & Zilversmit, D.B. (a) (1988)]. Treatment of these rabbits with mevinolin reduced CETA and plasma cholesterol but had no effect on the secretion of cholesterol therefore there is no regulatory effect on CETP by hepatic cholesterol secretion. Mevinolin had no direct effect on CETP.

10 Abnormal Lipoprotein Metabolism

1.10.1 Hypercholesterolaemia

Pattnaik *et al* [Pattnaik, N.M. *et al* (1978)] noticed that partially purified plasma from homozygous familial hypercholesterolaemics (FH) had 3-4 fold greater CETP specific activity than normal plasma. In 1984 Groener *et al* [Groener, J.E.M. *et al* (1984)] found that cholesteryl ester transfer activity (CETA) in delipidated plasma was 2 fold greater in hyperlipidaemic plasma than in normal.

More detailed studies were carried out to determine how CETA was affected by hypercholesterolaemia. Three models of hypercholesterolaemia: cholesterol fed rabbits, rabbits fed a casein/sucrose diet and Watanabe rabbits, were positively associated with CE/TG transfer activities [Son, Y-C. & Zilversmit, D.B. (1986)]. If the cholesterol fed rabbits were put back on a normal diet then the CETP reverted back to normal along with plasma cholesterol. When the time course of diet induced CETA increases were studied [Quig, D.W. & Zilversmit, D.B. (b) (1988)] it was found that CETA increased and reached a new steady state level within about 10 days with little change for up to 87 days even when plasma cholesterol continued to rise. The greatest increase in CETA occurred about five days after feeding. Low cholesterol and coconut oil (cholesterol free) diets were both associated with comparable increases in plasma cholesterol and CETA. A single high cholesterol or oil meal led to a twofold increase in plasma cholesterol but no change in CETA in 36 hours. Hypercholesterolaemia brought about by fasting lowered CETP activity not increased it and refeeding returned CETA to prefasting levels. In rats cholesterol feeding did not result in increased CETA.

1.10.2 Dyslipoproteinaemias

CETA has been measured in several cases of dyslipidaemia. Hyperbetalipoproteinaemics had decreased CETA [Fielding, P.E., Fielding, C.J. & Havel, R.J. (1983), Sparks, D.L. *et al* (1989)] as did dysbetalipoproteinaemics [Fielding, P.E. *et al* (1983)]. hypoalphalipoproteinaemics, familial combinded hyperlipoproteinaemics and familial hypercholesterolaemics [Sparkes, D.L. *et al* (1989)]. Contradictory results were found with hypertriglyceridaemics where patients with no sign of coronary heart disease had increased CETA and those with signs of disease had decreased CETA [Fielding, P.E. *et al* (1983)]. Another study found increased CETA in hypertriglyceridaemia [Sparkes, D.L. *et al* (1989)].

Differences in results from these experiments may be explained by the way in which the assays were carried out. Fielding measured mass transfer in whole plasma using endogenous substrates, a measurement which is more likely to be affected by substrate composition and concentration. Other groups, Zilversmit, Groener and Sparks, measured cholesteryl ester exchange, Zilversmit and Groener using exogenous lipoprotein substrates and Sparks using endogenous substrates bound to a solid phase.

Fielding's group found that the hyperlipidaemic, dyslipidaemic and hypertriglyceridaemic plasma could express normal activity using exogenous lipoproteins, therefore these patients did not have a deficiency of CETP. He proposed that the problem was the inability of the LDL/VLDL to accept cholesteryl ester. He also found in these cases that the net cholesterol transport between fibroblasts and medium was reversed i.e. the cells accumulated cholesterol despite normal or near normal cholesterol esterification in plasma.

Pappu and Illingworth added LPDS from abetalipoproteinaemic patients to normal plasma and found that it decreased normal CE and TG transfer. This inhibitory activity was heat insensitive and trypsin sensitive. They proposed that decreased CETA was due either to an increased production of inhibitor or that inhibitor normally associates with apoB containing lipoproteins and in abetalipoproteinaemics is found free.

Sparks *et al* found that the patients they studied all had an altered HDL composition comprising increased TG and decreased CE. Transfer to the HDL_3 fraction was reduced (but transfer to HDL_2 was increased in dyslipoproteinaemics and hypertriglyceridaemics). They concluded that impaired interaction of CETP with the HDL_3 pool may contribute to CHD risk in patients with plasma lipid abnormalities. They adhere to the idea that CETP is involved in equilibrating CE within the HDL subclasses prior to transfer to LDL/VLDL especially since CETP binds most avidly to HDL. Since binding of CETP is affected both by surface lipids and by apolipoprotein composition, changes in HDL structure may affect equilibration of CE. This impaired processing of HDL particles may prevent the formation of a particle that can be rapidly metabolised by the liver.

1.10.3 Hyperalphalipoproteinaemia

Familial hyperalphalipoproteinaemia (FHALP) is an inherited condition marked by high HDL cholesterol levels and longevity. Interest in this condition increased when two groups published investigations into the CETP activity from these plasmas. Koizumi *et al* [Koizumi, J. *et al* (1985)] studied two patients with HDL cholesterol levels of 7.83 and 4.52mM. The HDL were large with a high cholesterol:apoAI ratio. Transfer activity to VLDL and/or LDL could not be detected in either serum or the d>1.21 g/ml fraction of plasma using either exogenous or endogenous lipoproteins. Normal LPDS demonstrated normal CETA using FHALP lipoproteins as substrates. It was concluded that these patients had either a deficiency of CETP or an increased concentration of inhibitor.

Two further studies confirmed these initial reports. Kurasawa *et al* [Kurasawa, T. *et al* (1985)] studied a FHALP patient who also had hypertriglyceridaemia. Using endogenous lipoproteins to measure the activity it was found that the serum expressed low CETP activity. Unlike other HTGs this patient's HDL had low TG and high CE, a composition consistent with low CETA. Yamashita *et al* [Yamashita, S. *et al* (1987)] found that three patients with FHALP had no detectable CETA. Again HDL particle size was found to be increased and CE had accumulated in the HDL_2 fraction which was also apoE rich.

These findings were disputed [Groener, J.E.M., daCol, P.G. & Kostner, G.M. (1987)] when CETP activity was measured in an Italian FHALP family. Two types of assay were used to measure this activity: using fixed amounts of LDL and HDL after removal of endogenous lipoproteins with polyethyleneglycol precipitation and addition of exogenous lipoproteins; and using tracer ^3H-HDL_3 in whole plasma. They

found no major differences between family members with HDLs of >1.7mM, <1.7mM or with their spouses. Using the tracer method to measure activity in d>1.18 g/ml LPDP, low CETA was found. However if this was calculated in terms of exchange between HDL CE and VLDL/LDL CE then low transfer can be explained by the uneven substrate distribution found in these patients. It was concluded that there is no connection between FHALP and CETA. A strong negative correlation was seen between % CE transfer and HDL cholesterol content.

CETA was measured in a Japanese family [Takegoshi, T. *et al* (1988)]. The patient had one tenth normal CETA using an exogenous donor/acceptor assay. If the FHALP plasma is added to exogenous CETP then activity is inhibited therefore it is likely that the patient has increased inhibitor levels. If the LPDP was chromatographed on phenyl Sepharose then normal CETP activity could be measured. These studies then seem to confirm the initial investigations and suggest that the decreased CETA observed is due to increased expression of inhibitor. In two Japanese FHALP patients the absence of CETP was confirmed using monoclonal antibodies to measure CETP protein [Brown, M.L. *et al* (1989)]. It was also discovered that these patients had a point mutation in a splice site of the CETP premessenger RNA leading to abnormal splicing and deficiency of CETP. It is possible that FHALP is a heterogeneous condition. FHALP may be secondary to a number of different abnormalities e.g. reduced CETP protein, increased inhibitor or other unidentified factors. There is also a difference in degree of FHALP in the cases studied. HDL cholesterol levels for the Japanese families are in the range of about 4-8mM, whereas in the Italian family the high HDL group ranged from 2-5mM.

1.10.4 Diabetes

Plasma CETP activities were found to be altered in diabetes. Plasma from patients with non insulin dependent diabetes mellitus (NIDDM) had reduced CETA [Fielding, C.J. *et al* (1984)]. If normal LDL/VLDL was incubated with NIDDM plasma, CETA was normalised. Conversely, NIDDM plasma reduced CETA in normal plasma. Thus it seems that NIDDM LDL/VLDL is somehow deficient in its ability to carry out transfer reactions. The LDL/VLDL fraction from NIDDM plasma studied here had an increased FC:PL ratio and spontaneously lost FC to HDL (increased FC in lipoproteins caused a concentration dependent inhibition of CETA [Morton, R.E. (1988)]. This suggested that the decreased CETA in NIDDM is mediated by the increased FC content of these lipoproteins and in fact it was found that the block in CETA was inversely correlated with the FC/PL ratio in LDL/VLDL. Net transfer of cholesterol from LDL/VLDL to HDL was observed in NIDDM which indicated that there was a chemical potential gradient of free cholesterol. The size of this FC gradient was inversely correlated with CETA. Substitution of diabetic LDL/VLDL into normal plasma increased the FC gradient for normal plasma, and substitution of normal LDL/VLDL into diabetic plasma decreased the FC gradient. Dysbetalipoproteinaemics and hyperbetalipoproteinaemics also have an increased FC/PL ratio.

However, CETA was completely normalised in diabetics by normal LDL/VLDL when the FC gradient was only partly reduced so there may be an additional abnormality of HDL from NIDDM.

It was reported that in general the FC content of lipoproteins affects TG and CE transfer [Morton, R.E. (1988)]. Using *in vitro* modified lipoproteins (by incubating with FC/PC dispersions) it was shown that if VLDL or LDL have an increased FC content then CE transfer from these particles is decreased while TG transfer was unaffected. If HDL FC were increased then CE transfer was not changed but TG transfer was increased. These effects would lead to an increased mass transfer . Enrichment with FC allows VLDL to be a more efficient donor of TG. It is possible that FC alters the availability of CE or TG for transfer by affecting their relative solubilities at the surface of the lipoprotein. An increase in lipoprotein FC may indicate a systemic rise in FC and would stimulate net transfer of cholesterol out of HDL and hence from cells.

Patients with insulin dependent diabetes mellitus (IDDM) have a 2-4 fold increased CETA similar to nondiabetic hyperlipidaemic patients [Bagdade, J.D. *et al* (1987)]. If diabetic VLDL is incubated with control HDL and CETP source, CETA is increased. Therefore it seems that diabetic VLDL is altered functionally. The diabetics' VLDL had a decreased TG/PL ratio and this ratio was increased in the HDL fraction. These core lipid changes are consistent with the increased CETA *in vivo*.

1.10.5 Others

CETA has been studied in various other pathological states. Mendez *et al* [Mendez, A.J., Perez, G.O. & Hsia, S.L. (1988)] studied patients with uraemia receiving maintenance haemodialysis. Many of these patients have serum lipid and lipoprotein abnormalities and may be at risk for atherosclerosis. These patients had significantly lower CETA than controls. The patient VLDL/LDL fraction appeared to behave normally in transfer reactions therefore the defect appears to be in the d>1.063g/ml fraction. CETA found in the d>1.25g/ml fraction was lower in patients. Inhibitor was separated from CETP by passage over phenyl Sepharose and it was found that the recovery of CETA from patient plasma was two times that of the control. It is possible that CETP inhibitor is increased in these patients. In end stage renal disease [Dieplinger, H., Schoenfield, P.Y. & Fielding, C.J. (1986)] patients on haemodialysis show decreased transfer of CE to LDL/VLDL and low CETA compared with controls. The LDL/VLDL fraction had increased FC and inhibited CETA when combined with normal plasma similar to the effects seen in NIDDM. The investigators suggest that saturation of FC at the surface of LDL/VLDL causes inhibition of CETP. Finally Gupta *et al* [Gupta, A.K. *et al* (1989)], measuring the low rate of CE transfer in rats, showed that hypothyroid rats had significantly lower CETA than control rats. Hypothyroidism is associated with increased atherosclerotic risk.

11 Inhibition of CETP Activity by Antibodies

Both polyclonal and monoclonal antibodies have been developed to CETP. Monoclonal antibodies to human CETP have been used to verify that mass cholesteryl ester transfer is carried out by CETP in plasma *in vitro* [Yen, F.T. *et al* (1989)]. It was also found that inhibition of CETP did not affect cholesterol esterification i.e. LCAT is not controlled by CETP activity and therefore CETP may not regulate efflux of cholesterol from cells. Cholesterol was esterified in both HDL and LDL under conditions where CETP was inhibited indicating that both these lipoproteins act as substrates for LCAT. Initially the cholesterol esterification rate was four times higher in HDL, but this rate decreased after 6-8 hours while the esterification in LDL continued to increase. There was a strong positive correlation between transfer of CE to VLDL and VLDL TG. Thus the mass of TG in VLDL may be a major determinant of lipid mass transfer. The rate of transfer of CE to TG rich lipoproteins may influence the rate of delivery of cholesterol to the liver.

Two studies were carried out looking at the effect of inhibiting CETP activity *in vivo* in the rabbit. The first used a monoclonal antibody and attained 71% inhibition of CETP activity in rabbit plasma falling to 45% after 48 hours [Whitlock, M.E. *et al* (1989)]. Plasma total CE rose, largely reflected by an increase in HDL CE. HDL CE increased twofold and HDL TG fell reciprocally but amount of HDL protein did not change. This suggests that a CE for TG exchange process takes place in plasma. VLDL CE/TG ratio decreased. The rate of cholesterol esterification was not affected therefore the increase in HDL CE is probably due to decreased CE catabolism. Inhibition of CETP was also found to delay the clearance of ^{3}H-cholesteryl ether both from HDL particles and from the plasma compartment as a whole. There was a trend toward decreased radiolabel uptake in the liver after CETP inhibition but this was not significant. There was a negative correlation between liver uptake of radiolabel and the amount of CE in HDL. Thus it seems that *in vivo* CETP is important in CE metabolism by promoting CE exchange for TG and promoting clearance of CE from plasma probably by transferring CE to VLDL which is then catabolised by the liver or by a direct effect on hepatocyte uptake of CE. The authors suggest a regulatory role for CETP in reverse cholesterol transport. These inhibition studies are consistent with hyperalphalipoproteinaemia where patients also have large CE rich HDL and lack CETP.

The second study of CETP inhibition in rabbits showed similar results [Abbey, M. & Calvert, G.D. (1989)]. They used a polyclonal antibody and were able to block CETP activity completely for 48 hours. They found similar changes in HDL CE and TG and also found an increased HDL particle size. LCAT activity did not change in this study either and HDL conversion protein activity, an activity capable of converting HDL_3 to both larger and smaller particles [Rye, K. & Barter, P.J. (1986)], was not changed. No significant changes were detected in LDL or VLDL. Control animals had a significantly decreased proportion of cholesterol in HDL and an increased proportion in LDL. The inhibited animals had the converse. This indicates that the blocked animals had a less

atherogenic distribution of cholesterol in their lipoproteins. This suggested atherogenic role for CETP may only be important when plasma cholesterol is elevated. Plasma CETP levels in the rabbit needed to return to only 20-30% of the normal plasma CETP levels in order to get normalisation of the effects (in fact about the level found in human plasma). This indicates that the CETP level in rabbits is 3-5 times higher than necessary to have maximum effect.

12 Injection of CETP into Non-CETP Animals

In an *in vitro* study, rabbit CETP was incubated with pig plasma [Abbey, M., Calvert, G.D. & Barter, P.J. (1984)]. VLDL CE increased and TG decreased. LDL and HDL TG increased. As there was more apoE in LDL, then either CETP transfers apoE or apoE transfer is secondary to TG transfer.

The converse of the antibody inhibition studies, i.e. the addition of CETP to a CETP free animal, was carried out in rats. These studies were hampered by the short half life of CETP in rats. Partially purified CETP was injected into rats up to a level similar to that found in rabbits [Quig, D.W. & Zilversmit, D.B. (1986)]. Seventy percent of activity was lost in 6 hours. There was net transfer of CE out of HDL but no change in total cholesterol or the proportion of total cholesterol that was esterified. No effects were seen after 24 hours. All the changes observed here were transient probably due to the high turnover of lipoproteins in the rat.

Groener *et al* [Groener, J.E.M., van Gent, T. & van Tol, A. (1989)] also injected partially purified human CETP into rats to give levels between 1.5-4 times that seen in human plasma for 6 hours. They observed a biphasic decay of activity. At 6 hours (there was no change between 6 and 24 hours) there was an increase in the amount of apoB but no change in serum total cholesterol, free cholesterol, TG, apo AI, apoE or apoAIV. VLDL and LDL had increased total cholesterol and there was a decrease of total cholesterol in the large apoE rich HDL fraction. Smaller apoAI containing particles appeared and the mean diameter of HDL decreased. ApoE which is normally only found on the large HDL fraction was redistributed to smaller particles. The clearance of ^{3}H-cholesteryl ether apoAI rich HDL particles was increased in CETP treated rats, their half life decreasing from 3.9 to 2.2 hours. This was accompanied by an increased accumulation of radiolabel in the liver in treated rats. This may be explained by the redistribution of cholesterol to VLDL and LDL which may be cleared by the liver. In the control at time zero, the radiolabel was found on apoAI rich particles but after 6 hours it was found on the larger apoE rich HDL. This may indicate that the apoAI rich HDL are precursors for apoE rich HDL. In the treated rats label was found evenly distributed between all lipoprotein fraction at 6 hours.

13 Physiological Roles of CETP

The exact physiological role of CETP and its involvement in the development or prevention of atherosclerosis is unknown. There is evidence for the involvement of CETP in the progression of CHD and that decreased expression of the enzyme is beneficial.

In cases predisposing to atherosclerosis e.g. hyperlipidaemia [Son, Y.C. & Zilversmit, D.B. (1986)] and IDDM [Bagdade, J.B. *et al* (1987)], plasma CETP levels are increased and in a condition associated with longevity, FHALP, plasma CETP levels may be reduced or negligible [Koizumi, J. *et al* (1985), Takegoshi, T. *et al* (1988)]. There is also the circumstantial evidence from plasma levels of CETP found in certain species. Species which are at high risk of CHD e.g. humans and rabbits, have high plasma levels of CETP whereas those species which seem to be relatively resistant to CHD e.g. rats and dogs, have low levels of plasma CETP [Ha, Y.C. & Barter, P.J. (1982)].

However there is contradictory evidence for its involvement in CHD. In NIDDM [Fielding, C.J. *et al* (1984)], in some dyslipidaemic states [Fielding, P.E. *et al* (1983), Sparks, D.L. *et al* (1989)] and in uraemia [Mendez, A.J. *et al* (1988), Dieplinger, H. *et al* (1986)] there is an increased risk for CHD but decreased levels of plasma CETP. The pig is a species which has low levels of CETP but is susceptible to atherosclerosis. In FH patients who have xanthomas, regression has been shown to occur after treatment with probucol, a drug that results in the increase of plasma CETP activity [Matsuzawa, Y. *et al* (1988), Sirtori, C.R. *et al* (1988)].

Thus the physiological role of CETP is undefined. Evidence for its participation in various processes is often contrary and inconclusive. CETP may be involved in; reverse cholesterol transport (RCT), lipoprotein remodelling, equilibration of CE and TG between lipoprotein classes and processing of post prandial lipids.

RCT is the process by which cholesterol is returned from peripheral cells to the liver for excretion. There are four stages: picking up cholesterol from peripheral cells, esterification of this cholesterol by LCAT, transfer to other lipoproteins and delivery to the liver. CETP may play a role at any of these stages. Since CE and TG efflux from macrophages is stimulated by CETP [Morton, R.E. (b) (1988)] it appears that CETP can directly influence CE efflux from peripheral cells. HDL can act as an acceptor of cellular cholesterol [Oram, J.F. *et al* (1981)]. CETP exists in plasma as a complex with LCAT, an enzyme involved in esterifying plasma cholesterol [Fielding, P.E. and Fielding, C.J. (1980), Francome, O.J. *et al* (1989)]. The action of LCAT may be important in 'fixing' cholesterol which has been picked up from cells by HDL, in the plasma compartment [Glomset, J.A. (1968)]. HDL is the preferred substrate for LCAT, though LDL has been shown to act as a substrate over long incubations [Yen, F.T. *et al* (1989)]. Most of the CE in plasma is LCAT derived. It has also been suggested that CETP relieves substrate inhibition of LCAT by transferring CE that accumulates in HDL, reducing cholesterol concentration in the acceptor and leading to increased flow of cholesterol out of the cell [Chajek, T., Aron, L. & Fielding, C.J. (1980), Fielding, C.J. & Fielding, P.J. (1981)]. However there is now evidence that LCAT activity is independent from CETP activity [Stein, O. *et al* (1986), Yen, F.T. *et al* (1989)] and relief of substrate inhibition may only be important in static systems e.g. cell culture. *In vivo* the free cholesterol gradient is probably maintained by turnover of lipoproteins.

If macrophages (either J774s or human monocyte derived macrophages) were allowed to accumulate labelled CE and TG by incubating them with ^{3}H-oleate/albumin, and then incubated with CETP, then CE and TG efflux to the medium [Morton, R.E. (b) (1988)]. The CE mass of the cell was decreased so there was a net transfer of lipid. A lipoprotein acceptor was not required for the effect, though LDL and HDL did stimulate efflux but only at high concentration. Label did not transfer from lipoproteins to the cells regardless of whether the cells were cholesterol loaded or not. This may be due to inhibition of uptake but it is unlikely since HDL and LDL are taken up by different receptors. Secreted lipids were associated with large molecular weight components of d>1.21g/ml and were not associated with CETP. The lipids were readily incorporated into HDL or LDL during or after incubation. CE were transferred in preference to TG. The transfer mechanism requires no cell energy, active protein synthesis or trypsin sensitive protein.

There is controversy over which lipoprotein(s) are responsible for delivering cholesterol back to the liver. If HDL is the main lipoprotein responsible for delivering cholesterol back to the liver for excretion [Glomset, J.A. (1968), Tall, A.R. & Small, D.M. (1980)], then CETP would not be involved in this part of RCT and indeed would divert cholesterol away from this pathway by transferring CE to LDL/VLDL which may be redirected to other pools in the liver or may be delivered back to the periphery. CETP may promote RCT by increasing the flux of CE to LDL/VLDL, which in turn may be taken up by the liver. LDL and VLDL remnants are undoubtedly taken up by the liver [Goldstein, J.L. & Brown, M.S. (1975), Norum, K.R. *et al* (1983)] but it has been argued that different cholesterol pools exist in the liver and that HDL is responsible for delivering cholesterol to that pool destined for bile acid synthesis and secretion. If LDL/VLDL are responsible for delivering cholesterol back to the liver for excretion [Gotto, A.M. Jr., Pownall, H.J. & Havel P.J. (1986), Fielding, P.E., Fielding, C.J. & Havel, R.J. (1983), Havel, R.J. (1985)], then CETP is an integral component of RCT. The rate of transfer of CE to lower density lipoproteins is similar to the rate of cholesterol esterification. CETP has also been shown to increase the CE uptake by HepG2s in culture [Granot, E., Tabas, I. & Tall, A.R. (1987)] though this has been disputed [Rinniger, F. & Pittman, R.C. (1989)]. In cases where CETP activity has been inhibited by antibodies in the rabbit [Whitlock, M.E. *et al* (1989)], inhibition of CETP was found to delay the clearance of ^{3}H-cholesteryl linoleyl ether from HDL and whole plasma and to decrease the appearance of radiolabel in the liver. Addition of CETP to rats increased the clearance of ^{3}H-cholesteryl linoleyl ether labelled apo-AI rich HDL from the plasma and increased radiolabel accumulation in the liver [Groener, J.E.M. *et al* (1989)]. This evidence implies that CETP is involved in RCT and increases the clearance of cholesterol from the plasma.

Granot *et al* [Granot, E. *et al* (1987)] proposed that CETP may be involved in cellular uptake of cholesteryl esters. They incubated HepG2 cells with ^{3}H-CE labelled HDL_3 and increasing amounts of CETP (either d>1.21g/ml plasma, partially purified or purified protein) and found a progressive increase in CE uptake, to about threefold.

This process was saturated by both CETP and HDL at relatively low concentrations. The uptake increased over 18 hours and protein uptake was not affected. An increased amount of labelled cholesterol was found in the cells which indicates that the CE was taken up and also degraded. CE degradation was inhibited by chloroquine. This effect was also seen in smooth muscle cells and to a lesser extent in fibroblasts but not in endothelial cells or J774s. Immunoprecipitation of CETP abolished the effect. In incubations without CETP, 0.7% of label was transferred to d<1.063 g/ml plasma and this was increased to 1.8% in the presence of CETP. After an 18 hour incubation, the d<1.063 fraction was isolated and reincubated with new HepG2 cells. There was no accumulation of radioactivity into the cells. Therefore although transfer of label to d<1.063g/ml particles did take place it is unlikely that this accounts for the accumulation of labelled CE by the cells by uptake of apoB containing particles. Preincubation of the cells with LDL, which would cause a downregulation of LDL receptor, decreased the absolute amount of HDL CE uptake but did not affect the stimulation by CETP.

Rinniger and Pittman [Rinniger, F. & Pittman, R.C. (1989)] disagreed with the conclusions reached by Granot *et al*. They saw the CETP stimulated uptake of CE from ^{3}H-HDL_3 in HepG2s in long incubations. However the effect was not seen in shorter, 4 hour, incubations nor in fibroblasts. This suggested that components secreted by the cells in culture e.g. lipoproteins were necessary for the effect. If CE were transferred to lower density lipoproteins and then these were taken up by receptors then this would explain the effect. To investigate this they used four inhibitors of LDL uptake: heparin, monensin, LDL receptor antibodies and anti-apoB and E binding domains. All these agents blocked CETP stimulated uptake of CE. Heparin increased the amount of labelled CE in the d<1.063g/ml fraction. Granot's study concluded that not enough CE was transferred to d<1.063g/ml particles in order to account for the effect. However they looked at the particles left at the end of the experiment which were those which were not preferentially taken up. Also since down regulation does not completely abolish the amount of receptor expressed if the labelled particles were of high affinity they would still be taken up.

Lipoprotein remodelling is a process in which CETP may be involved. Decklebaum *et al* [Decklebaum, R.J. *et al* (1988)] studied the remodelling of lipoproteins in vitro. Transfer of CE from LDL to VLDL and TG back to LDL was detected. The TG in LDL could be hydrolysed by lipoprotein lipase. Using a cyclic treatment of HDL_2 with CETP, causing loss of CE to VLDL and acquisition of TG, and then lipoprotein lipase, which hydrolyses the accummulated TGs, the HDL_2 could be converted to HDL_3. The relative amounts of VLDL and LDL/HDL would influence lipid exchanges and hence remodelling. CETP could be involved in VLDL to LDL interconversions. CE transfer from VLDL would result in replacement by TG. If this TG is hydrolysed by lipoprotein lipase then smaller particles will be formed. Abetalipoproteinaemic patients, who had decreased CETA, had an increased proportion of HDL_2 which ties in with the idea that transfer protein may be involved in converting HDL_2 into HDL_3 [Pappu, A.S. & Illingworth, D.R. (1988)]. FH patients who have been treated with probucol [Sirtori,

C.R. *et al* (1988)] have increased CETA and decreased HDL_2 while the HDL_3 fraction is unchanged. This supports the theory that CETP is involved in HDL particle interconversion.

CETP shows substrate specificity for species of CE [Morton, R.E. (1986)]. Differences in rates of transfer according to acyl chain would lead to different CE compositions of lipoproteins. These differences may influence the metabolism of lipoprotein particles e.g. enrichment with saturated CE would decrease the fluidity of the lipoprotein particle which may affect further transfers or remodelling reactions. CETP may be involved in equilibrating CE within HDL subclasses by carrying out particle interconversions prior to transfer to lower density lipoproteins. Impaired processing of HDL may also prevent the formation of a particle that can be rapidly taken up by the liver.

CETP may be involved in the post prandial processing of lipids. Lipolysis of lipoprotein particles leads to increased binding of CETP and an increase in its activity [Castro, G. & Fielding, C.J. (1988), Sammett. D. & Tall, A.R. (1985)]. CETA increases post prandially [Tall, A. *et al* (1986)] and also Caco2 cells can secrete CETP in a way that is regulated by fatty acids [Faust, R.A. & Albers, J.J. (1988)]. This evidence suggests that CETP secretion could be upregulated in both acute and chronic lipaemia and CETP may be invoved in both states.

This project was started in January 1987. At that time much fundamental knowledge about CETP was lacking which was rapidly provided in the intervening years. Therefore many of the experiments originally laid out in the aims of the project have been superceded and some areas of investigation were introduced as more information became available. At the time of embarking on this project the CETP protein had not been well characterised to the 74kD form, and no good antibodies with which to carry out *in vivo* inhibition studies were available. LTIP was not characterised until 1989 and up until then its existence was putative. Only macrophages had been reported to secrete CETP. The CETP gene was cloned and sequenced only in 1987.

The aim of this project was to investigate further the role of CETP. A number of approaches were taken:-

1/ CETP activity in plasma is the net result of a number of influencing factors including CETP mass, concentration of inhibitor and composition and concentration of substrate lipoproteins. This last variable can be eliminated by using exogenous substrates in an activity assay. It was hoped that by measuring CETA *per se* in plasma, i.e. the net result of inhibitor and CETP, and then by measuring CETP protein mass, using antibodies, that the relationship between CETP and its inhibitor LTIP as affecting expressed plasma CETA could be investigated.

2/ Inhibition of CETP activity *in vivo* is a key experiment which would yield information on the role of CETP in the whole animal. In the absence of a good chemical inhibitor the best inhibitor to use would be antibodies, either polyclonal or monoclonal.

3/ There are drugs available that affect cholesterol metabolism *in vivo*. By looking at the effects of these agents both on plasma cholesterol and on plasma CETP activity, one might be able to infer what effects CETP is having on cholesterol metabolism.

4/ Cells in culture e.g. macrophages, J774, HepG2 and CaCo2 are known to secrete CETP. Fatty acids increase CETP secretion by CaCo2 cells. Identifying factors which may affect CETP secretion may shed light on what regulates plasma CETP levels *in vivo*.

5/ A negative correlation has been found between plasma HDL levels and plasma CETP activity. Since probes for the CETP gene are available it is possible to look for polymorphisms in the CETP gene and test whether there is any relationship between these polymorphisms and plasma CETP activity and HDL levels. If CETP has an effect on plasma HDL then one might expect to find a relationship between any detected polymorphism and plasma HDL levels.

CHAPTER II

METHODS

1 General Methods

2.1.1 Buffers

Phosphate buffered saline (PBS) - 80g NaCl (137mM), 2g KCl (2.7mM), 2g KH_2PO_4 (1.5mM), 11.5g Na_2HPO_4 (8.1mM) in 10L distilled water.

Tris saline buffer pH 7.4 - 0.15M NaCl, 10mM Tris, 1mM EDTA, 0.05% NaN_3.

2.1.2 Analytical Procedures

Total cholesterol was measured using an enzymic colorimetric assay (Cat No 816302, Boehringer Mannheim GmbH). The same enzymic test was used to measure HDL cholesterol after precipitation of plasma LDL and VLDL by heparin manganese [Burnstein, M., Scholnick, H.R. & Morfin, R. (1970)]. Triglycerides were measured using an enzymic colorimetric test (Cat No 816370 Boehringer Mannhein GmbH, or Baker Instruments). Tests were carried either automatically (Centrifichem) or manually. Free fatty acids were measured using an enzymatic colorimetric kit (Wako, Alpha Laboratories). Phospholipids were measured as described [Dittmer J.C. & Wells M.A. (1969)]. If tissue or lipoprotein samples were used these were first extracted with one volume of chloroform:methanol 2:1 for 1 hour followed by addition of four volumes of 0.05M $CaCl_2$ to separate the phases [Folch, J., Lees, M. & Stanley, G.H.S. (1957)]. Protein concentrations were determined with Biorad Protein Assay according to the manufacturers instructions using bovine serum albumin as a reference standard.

For a more sensitive measurement of cholesterol a spectrofluorometric technique was used [Heider J.G. & Boyett R.L. (1978)] with the following differences which improved reproducibility in our hands. The 0.05M sodium phosphate buffer pH 7.0 contained PEG 6000 instead of Carbowax and 10mM sodium taurocholate was present in both the free and total cholesterol reagent. Cholesterol esterase at 0.8 IU/ml was used in the total cholesterol reagent. Standards and samples were diluted in propan-2-ol to a volume of 50µl and 1ml of reagent was added. Tubes were incubated for 30 minutes at 37°C. Fluoresence was measured using a Perkin-Elmer LS-5B luminescence spectrometer at excitation wavelength 325nm and emission wavelength 415nm.

2.1.3 Preparation of Lipoproteins and LPDS/LPDP

Lipoprotein fractions were prepared by differential ultracentrifugation at different densities. Chylomicrons (CM) were separated by flotation at 1.0063g/ml, very low density lipoprotein (VLDL) by flotation at 1.0063g/ml, low density lipoprotein (LDL) at 1.063g/ml, and high density lipoprotein (HDL) at 1.21g/ml [Schumaker, V.N. & Puppione, D.L. (1986)]. HDL can be divided into two subfractions; HDL_2 by flotation at 1.125g/ml and HDL_3 by flotation at 1.215g/ml after removal of the HDL_2 subfraction [Anderson, D.W. *et al* (1978)]. Final densities were achieved by adding appropriate amounts of density solutions. Several stock density solutions were made:- solution 1 d=1.0063g/ml (34.38g NaCl + 3000ml H_2O), solution 4 d=1.3104g/ml (115.34g NaBr + 250ml solution 1), solution 5 d=1.2160g/ml (75.82g NaBr + 250ml solution 1), solution 6 d=1.1816g/ml (63.30g NaBr + 250 ml solution 1), solution 7 d=1.0670g/ml (20.37g NaBr + 250ml solution 1) and solution 8 d=1.4744g/ml (193.8g NaBr + 250ml solution 1). Densities of stock solutions were measured using a Paar DMA46 densitometer (Stanton Redcroft).

The main lipoprotein subclasses were separated sequentially as follows. For large scale preparations either 25ml polycarbonate bottles were used in a Beckman 55.2 Ti rotor or 70ml polycarbonate bottles were used in a Beckman 45 Ti rotor (but in this rotor the maximum speed for separation of LDL and HDL was only 45000rpm) in a Beckman L8-70M ultracentrifuge. For small preparations either 1ml or 3ml samples were centrifuged in a Beckman TL100 ultracentrifuge using a TLA 100.2 or a TLA 100.3 rotor respectively.

Lipoproteins were prepared at 20°*C* from either serum or plasma. To separate CM, tubes are two thirds filled, overlaid with solution 1 and centrifuged at 100000g for 20 minutes. The top one sixth volume is removed using a syringe and blunt needle and a further one sixth volume is removed as waste. VLDL was collected by overlaying the infranatant with solution 1 and centrifuging at 160000g for 20 hours (45000rpm) in L8-70M or at 350000g for 3 hours (100000rpm) in the TL100. The VLDL was removed as the top one sixth volume and waste was removed as before. The density was increased to 1.063g/ml by adding one half the volume of solution 6 to the resuspended supernatant. Any space was overlaid with solution 7. The tubes were then centrifuged as for the VLDL. LDL was harvested and the waste removed as before. The density was increased to 1.21g/ml by adding one half the volume of solution 8 to the resuspended supernatant and overlaying any space in the tube with solution 5. This was centrifuged at 200000g for 24 hours (50000rpm) in the L8-70M or 350000g for 3 hours (100000rpm) in the TL100 centrifuge. HDL was harvested as the top one sixth volume and one sixth volume was removed as waste. The remaining infranatant was lipoprotein depleted plasma (LPDP) or lipoprotein depleted serum (LPDS) depending on the starting material.

To prepare the HDL subfractions, the lower density lipoproteins were firstly removed by flotation at 1.063g/ml. The background density was increased to 1.125g/ml by adding one quarter the volume of solution 4. HDL_2 was isolated by flotation at 1.125g/ml at 200000g (50000rpm) for 24 hours in a Beckman L8-70M ultracentrifuge. After harvesting HDL_2 the density was increased to 1.215g/ml by the addition of an equal volume of solution 4. Again the tubes were centrifuged at 200000g (50000rpm) for 24 hours. HDL_3 can be harvested from the top of the tube and after the removal of waste, LPDP or LPDS remains.

LPDP or LPDS could be obtained in a one step ultracentrifugation at d=1.215g/ml. This final density was obtained by adding two volumes of solution 4 to one volume of plasma or serum. This was then centrifuged at 200000g (50000rpm) for 24 hours in a Beckman L8-70M ultracentrifuge or 350000g (100000rpm) for 3 hours in a TL100 centrifuge. After centrifugation the top one third volume was removed and the infranatant resuspended.

After preparation, lipoproteins or LPDP/S were dialysed against 20 volumes of Tris saline buffer and then sterilised by passage through a 0.2 micron filter.

2.1.4 Electrophoretic Techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out according to the method of Laemmli [Laemmli U.K. (1970)]. Briefly, 1M Tris pH 8.7 (10ml) was mixed with an appropriate volume of acrylamide 30%: bisacrylamide 0.8% to give the final desired concentration of acrylamide after adding water to bring the final volume to 40ml. 20% SDS (200µl) was added followed by N,N,N',N',-tetramethylethylenediamine (TEMED) (20µl) and freshly prepared 13% ammonium persulphate (100µl). Once poured in the mold the gel was overlaid with water-saturated isobutanol which was rinsed off when the gel had set. The stacking gel consisted of 5.25% acrylamide. Acrylamide 30%: bisacrylamide 0.8% (3.5ml) was mixed with 0.83M Tris HCl pH 6.8 (3.5ml) and water (12.8ml). 20% SDS (100µl), TEMED (10µl) and 13% ammonium persulphate (100µl) were then added. Samples for electrophoresis were prepared in sample buffer, 20% SDS (5ml) 0.83M Tris HCl pH 6.8 (1.5ml) glycerol (5ml) and water (38.5ml). Bromophenol blue (1µg/ml) was added as a dye. Before use, sample buffer was saturated with urea and 2-mercaptoethanol (final concentration 5%) was added. No less than a 1/2 dilution of sample buffer was made by adding sample. Gels were run overnight at 35V in running buffer (5 x concentrated running buffer = glycine 144g/l, Tris 30g/l, 20% SDS 25ml/l).

Lipofilm kits (Sebia, supplied by Labmedics) were used to analyse lipoproteins electrophoretically. The gels were run according to the manufacturer's instructions. Samples were prestained for 30 minutes in Sudan Black prior to electrophoresis as described in the instructions.

2.1.5 Autoradiography

DNA and RNA blots or air dried Lipofilms were autoradiographed at -20°C. Films were developed in Kodak LX24 developer for 1 to 3 minutes until sufficient reaction had occured, then stopped in 1% acetic acid for one minute and finally fixed for 3 minutes in Kodak FX40 fixer. The films were then washed in water.

2.1.6 Stains

For Coomassie blue staining gels were first fixed for 30 minutes in 10% acetic acid, 50% methanol. They were then stained for 5 minutes in 0.6% Coomassie brilliant blue R, 10% acetic acid, 50% methanol and then destained in 10% methanol, 10% acetic acid until the background was clear.

Silver staining was carried out according to the method of Morrissey [Morrissey, J.H. (1981)]. Gels were prefixed in 50% methanol, 10% acetic acid for 30 minutes and then 5% methanol, 7% acetic acid for 30 minutes. Fixing was in 10% glutaraldehyde for 30 minutes. Gels were then rinsed in distilled water and soaked over 2 to 3 hours in several changes of distilled water. After washing the gels were then soaked firstly in dithiothreitol (5μg/ml) and then 0.1% silver nitrate, each for 30 minutes. Next the gels were rinsed quickly two times with distilled water and once with a small amount of developer (50μl of 37% formaldehyde in 100ml 3% sodium bicarbonate). Gels were then soaked in developer with mixing until the desired staining was reached. Development was stopped by adding 2.3M citric acid (5ml) and leaving for 10 minutes. Finally gels were washed several times with distilled water.

Protein on nitrocellulose was stained with Ponceau Red S, 0.05% in 3% trichloroacetic acid, for 5 minutes and then the filter was washed in distilled water. Staining could be totally removed by destaining in PBS.

2 Enzyme Assays

2.2.1 CETP Assay

^{3}H-HDL$_3$ donor particles were prepared by a modified method of Morton and Zilversmit [Morton, R.E. & Zilversmit, D.B. (1981b)]. Blood was collected in disodium EDTA (final concentration 1mg/ml EDTA) and the plasma separated by centrifugation (2800rpm, 1200g, 15 minutes). The d>1.125g/ml fraction of plasma was isolated by centrifugation as described previously. Less dense (d<1.125g/ml) lipoproteins were quantitatively removed using a syringe and the infranatant dialysed against Tris-saline buffer.

A mixture of 9μl of triolein in hexane (10mg/ml), 15μl egg lecithin phosphatidyl choline in hexane (100mg/ml), 8.8μl of 1mM butylated hydroxytoluene in chloroform, 160μl of ^{3}H-cholesteryl oleate in toluene (160μCi of a stock at 45Ci/mmol) and 184μl of cholesteryl oleate in hexane (1mg/ml) was dried down in a siliconised glass tube under a stream of nitrogen. To label the lipoproteins with a different isotope or to

label a different moiety the ^{3}H-cholesteryl oleate was replaced with ^{14}C-cholesteryl oleate (20μCi), ^{14}C-triolein (20μCi) or to double label, ^{3}H-cholesteryl oleate and ^{14}C-triolein were added together. An aliquot of 0.8ml of freshly prepared Tris-EDTA buffer (50mM Tris-HCl, pH7.4 and 0.01% disodium EDTA) was added to the mixture and the tube flushed with nitrogen. It was then covered with Nescofilm and mixed on a vortex for 10 minutes to give a cloudy suspension. Sonication of this in a MSE Soniprep 150 using a 3mm probe (2 x 20 minutes, 2 microns amplitude) produced a clear solution which was transferred to a siliconised glass conical flask together with 20ml of dialysed d>1.125g/ml plasma infranatant, 1.66ml of 14mM DTNB and 0.2ml of 0.4M disodium EDTA containing 4g/100ml NaN_3. The mixture was incubated for 18 hours at 37°C under nitrogen. ^{3}H-HDL_3 was subsequently isolated by flotation at d=1.215g/ml and further purified by a second centrifugation at d=1.125g/ml. The infranatant from this was then centrifuged once again at d=1.215g/ml and the ^{3}H-HDL_3 in the supernatant harvested. The labelled lipoprotein fraction was loaded onto a 80ml (1.5 x 45cm) Sepharose CL-4B gel filtration column and eluted overnight with Tris-saline buffer. Samples of the fractions were counted by liquid scintillation counting and the radioactive peak pooled. In earlier experiments either the final two ultracentrifugation steps or the Sepharose CL-4B gel filtration step were omitted. Details are given in the figure legends. To label different lipoprotein fractions the lipid emulsion could be incubated with either whole plasma or LPDP plus the lipoprotein of choice and then subsequently isolated at the correct density. Labelled lipoproteins were stored at 4°C after sterilisation by passing through a 0.2 micron filter.

A combined LDL/VLDL fraction (d<1.063g/ml) from pooled normal plasma was used as a transfer acceptor. This was isolated by ultracentrifugation at d=1.063g/ml after removal of chylomicrons as described previously. The fraction was purified by a further flotation at d=1.063g/ml, dialysed against Tris-saline buffer and stored at 4°C after sterilisation by passing through a 0.2 micron filter. In some experiments the second ultracentrifugation was omitted. Details are given in the figure legends.

When initially testing for the optimum precipitation conditions dilutions of a stock heparin manganese solution (2500 U/ml heparin (Sigma cat. no. 9133): 1M manganese chloride) were used. Once the best precipitation conditions were found (described in Chapter III), 2000 U/ml heparin:0.8M manganese chloride solution (25μl) was routinely used to precipitate lipoproteins in a 60μl assay, giving final concentrations of 213U/ml heparin and 0.085M $MnCl_2$.

^{3}H-HDL_3 (5μg protein) and LDL/VLDL (8μg protein) were incubated together in the presence of 0.7mM DTNB with the d>1.215g/ml fraction of test plasma (LPDP) in a total volume of 60μl. The DTNB was added as 5μl of a 8.4mM solution, pH 7.0. The assay was carried out in microtitre plates which had been previously coated by incubating in 3% BSA for one hour at 42°C, shaking off excess solution and air drying. Tris-saline buffer was used for the assay and all LPDP samples had been previously dialysed against this buffer. It is essential that samples assayed do not

contain phosphate as this may interfere with precipitation since manganese phosphate can precipitate lipoproteins. Plates were incubated at 42°C for 2.5 hours with shaking. The assay was stopped by placing the plate on ice. Chilled plasma (150μl) was added to each well with mixing, followed by 25μl of heparin manganese reagent (heparin 2000 U/ml in 0.8M $MnCl_2$) [Tollefson, J.H. & Albers, J.J. (1986)]. The plate was left to stand for 25 minutes on ice. The precipitate was pelleted by centrifugation (2800rpm, 1200g, 25 minutes, 4°C) and ^{3}H-radioactivity measured in 150μl of supernatant. Activity was expressed as percentage transfer (from supernatant to pellet) per 2.5 hours. This was linear to 35% transfer. The protein content of each LPDP sample was determined by the Bio-Rad Protein Assay (Bio-rad U.K.) and specific activity expressed as percentage transfer/μg LPDP protein/2.5 hours.

The above assay could be made more sensitive by incubating for 8 hours at 42°C. If assays were carried out in this way the assay volume was doubled so that the assay could be carried out in larger tubes thus avoiding problems with evaporation encountered when incubating microtitre plates for the longer period of time; 10μg of ^{3}H-HDL_3 was incubated with 16μg LDL/VLDL. Assay buffer containing 6% BSA was added to the assay to give a final BSA concentration of 0.5%. Assays were carried out in a volume of 120μl in microfuge tubes and incubated in a shaking water bath. At the end of the incubation 300μl of cold plasma and 50μl heparin:manganese reagent (2000 U/ml heparin: 0.8M $MnCl_2$) were added. Precipitation was carried out in the usual way. Samples (300μl) of supernatant were counted.

When samples were to be assayed for their inhibitory effect on CETP activity, they were incubated in the usual CETP assay in the presence of a control amount of either human LPDS, human post CM52 CETP or rabbit post CM52 CETP. Enough protein was added to give a control activity of between 25 to 30% transfer. Inhibition was calculated as the percentage inhibition of this control activity.

The solid phase CETP assay was carried out as follows. ^{3}H-HDL was coupled to CNBr activated Sepharose (Pharmacia) according to the manufacturer's instructions. CNBr activated Sepharose (0.285g) was allowed to swell for 15 minutes in 1mM HCl and then washed with 5 x 20ml 1mM HCl. The gel was washed with 2.5ml coupling buffer (0.1M $NaHCO_3$ pH 8.3, 0.5M NaCl) and transferred to a solution of 8.8mg ^{3}H-HDL (2.7μCi) in coupling buffer. The solution was then mixed end over end for two hours at room temperature. The gel was transferred to blocking buffer (0.2M glycine pH 8.0) and left to stand for 2 hours at room temperature. Excess ligand was washed away with alternate washes of 4 x 10ml coupling buffer and 4 x 10ml acetate (0.1M acetate pH8.4). ^{3}H-HDL-Sepharose (10μl, 50000dpm) was incubated with 40μg LDL/VLDL in the presence of 0.7mM DTNB and LPDS in a volume of 200μl. At the end of the incubation, HDL-Sepharose was pelleted by centrifugation (2800rpm, 1200g, 15 minutes) and the supernatant was sampled (100μl) and counted by liquid scintillation counting.

2.2.2 LCAT Assay

Substrate micelles were made by drying down egg lecithin phosphatidyl choline (8µl of a 100mg/ml solution in hexane, equivalent to 0.9µmol) and [4-^{14}C] cholesterol (8.4µCi at 56mCi/mmol, equivalent to 0.15µmol) under a stream of nitrogen in a 5ml polypropylene tube (Sarstedt). One ml 'phosphate buffer' (50mM KH_2PO_4 pH7.4, 0.025% EDTA, 2mM NaN_3) was added and the tube mixed well. The resultant cloudy solution was sonicated (4 x 5 minute bursts at 2 micron amplitude) in a MSE Soniprep 150 using a 9mm probe. The resulting substrate vesicles contained approximately 80000-90000dpm per 5µl. Solutions could be stored at 4°*C* for 2 weeks without loss of activity.

A dialysed LPDP (d>1.215g/ml) fraction was used for measurement of LCAT activity. Twenty-five µl of substrate vesicles and 45µl of assay buffer (0.15g/l apoAI, 20g/l BSA, 8mM 2-mercaptoethanol in phosphate buffer) were mixed in a microfuge tube [Doi, Y. & Nishida, T. (1981)]. The tube was then flushed with nitrogen, capped and preincubated at 37°*C* for 30 minutes. The reaction was started with 20µl of an appropriate dilution of LPDP. This dilution was usually 1 in 2 in phosphate buffer and was chosen so that the substrate remained in excess during the reaction. Tubes were again flushed with nitrogen, capped, mixed and incubated for 45 minutes at 37°*C*. At the end of the incubation the assay was stopped by the addition of 750µl hexane/isopropanol 3:2 v/v followed by 100µl of 60% ethanol containing 0.02% Coomassie blue [Belfrage, P. & Vaughan, M. (1969)]. The tubes were thoroughly mixed on a vortex mixer and centrifuged (1750rpm for 3 minutes) to separate the phases. The upper organic colourless layer was sampled (350µl) and transferred to an HPLC autosampler vial. The samples were dried down and then dissolved in 1.25ml injection solvent (0.5g/l cholesterol, 0.5g/l cholesteryl oleate, 1g/l oleic acid in methanol:isopropanol 145:55 v/v). The cholesteryl ester content was determined by reversed-phase HPLC with radiochemical detection using a column of Spherisorb 5 micron ODS2 (15cm x 4.6mm id) and a mobile phase of propan-2-ol/methanol 55:45 v/v at a flow rate of 0.6ml/minute. The column eluant was mixed with Ecoscint scintillation fluid (National Diagnostics) in a 1:1 ratio prior to detection with a BETAcord 1208 radioactivity monitor (LKB). Two product cholesteryl ester peaks were separated and quantified against a ^{14}C-cholesteryl oleate standard curve, Dectection of cholesteryl ester was linear between sensitivity limits of 1000-10000 dpm/ml (8.13-81.3 pmol/ml).

3 Purificaton Techniques

2.3.1 CETP Purification

Partial purification was carried as described in previously published methods [Morton, R.E. & Zilversmit, D.B. (1982), Hesler, C.B. *et al* (1987)]. All purification steps were carried out at 4°*C*. LPDS or LPDP (200-300ml) was used as a starting material. This was adjusted to 4M NaCl by the addition of solid NaCl. If LPDS was

used directly after preparation by ultracentrifugation it was not dialysed but was adjusted to a final salt concentration of 4M taking into account the starting NaBr concentration. The LPDS was then centrifuged (35000rpm, 30 minutes, in a Beckman 45Ti rotor) before loading onto a 200ml phenyl Sepharose CL4B column, at 30 ml/hour, which has previously been equilibrated with 10mM Tris HCl pH 8.0, 2M NaCl. The column was then washed at 30ml/hour with 10mM Tris HCl pH 8.0, 0.15M NaCl until the absorbance of the eluant at 280nm was 0.3, usually overnight. CETP activity was eluted at 30ml/hour in distilled water, 0.02% NaN_3. Fractions were assayed and the active fractions pooled. This pool from the phenyl Sepharose column was adjusted to 50mM sodium acetate by the addition of 1/19 volume of 1M sodium acetate pH 4.5. The pool was then centrifuged (35000rpm, 30 minutes, in a Beckman 45Ti rotor). The supernatant was loaded onto a 200ml CM52 ion exchange column (Whatman) prequilibrated with 50mM sodium acetate, 0.02% NaN_3, 1mg/ml EDTA, pH 4.5. Activity was eluted at 30ml/hour with a 0-200mM NaCl gradient in sodium acetate buffer. The solutions used to form the linear gradient were 300ml sodium acetate, 0.02% NaN_3, 1mg/ml EDTA pH 4.5 and 300ml of the same buffer containing 200mM NaCl. Fractions (10ml) were collected into tubes containing 1ml 1M Tris phosphate pH 7.4 to neutralise the acetate buffer. Fractions were assayed and the peak of activity pooled, concentrated under nitrogen using an Amicon PM10 membrane and dialysed against Tris-saline buffer. This partially purified post CM52 fraction was stored at -20°*C*. Rabbit CETP was purified in an identical manner to human CETP.

The preparation of the lipid emulsion for CETP purification was according to the method of Hesler *et al* [Hesler, C.B. *et al* (1987)]. Egg lecithin phosphatidyl choline (60mg), triolein (300mg) and oleic acid (40mg) were mixed in a polypropylene tube with 3.5 μmoles of butylated hydroxytoluene. The solvents were evaporated under a stream of nitrogen for 2 hours. Solution 7 (d=1.063g/ml) (5ml) was added and the tube mixed. The mixture was then sonicated under nitrogen on ice in a MSE Soniprep 150 for 10x 15 second bursts at 2 micron amplitude at 3 minute intervals. The emulsion was then centrifuged (2800rpm, 1200g, 10 minutes) to remove any particulate matter. Emulsion (2ml) was put into a 5ml Beckman polyallomer tube and overlaid with 3ml solution 1, d=1.0063g/ml. The tubes were centrifuged (48000 rpm, 200000g, 1 hour) in a Beckman SW 50.1 rotor. After the centrifugation, the top 2ml were removed and loaded onto a 200ml Sepharose CL4B column. Emulsion was eluted in Tris-saline buffer. The milky emulsion peak was pooled and its composition assayed.

Post CM52 CETP (8mg) was incubated with either emulsion (12% phosphatidyl choline, 80% triolein, 9% oleic acid) or Intralipid 10% (KabiVitrum Ltd), (50mg). Mixes were incubated at 37°*C* under nitrogen for 30 minutes. The lipid CETP mixtures were then loaded onto a 100ml Sepharose CL4B column and eluted at 10 to 20 ml/hour with 50mM ammonium bicarbonate, 0.5M EDTA pH 8.5. Fractions (2ml) were collected and assayed for CETP activity and also examined by SDS PAGE.

CETP bound to Intralipid or emulsion was delipidated with ethanol; diethyl ether extraction [Hesler, C.B. *et al* (1987)] or with Cabosil (Kodak). Cabosil (1.5g) was stirred for 20 minutes at room temperature in 37.5ml acetone. This was then filtered through a sintered glass funnel. These last two steps were repeated. The washed Cabosil was transferred to a beaker and spread out over the bottom surface. The Cabosil was left so that the acetone could evaporate overnight and was then crushed to a fine powder with a mortar and pestle. For delipidation Cabosil (1.5g) was added to CETP bound to Intralipid (10.5ml) and stirred slowly overnight at 4°*C*. The slurry was centrifuged (17000rpm, 25000g, 60 minutes, 4°*C*) and the supernatant decanted and tested for CETP activity.

For the batch hydroxyapatite binding of post CM52 CETP [Jarnagin, A.S. *et al* (1987)] hydroxyapatite gel was washed two times with 3ml of 10mM Tris HCl pH7.4. At each wash, gel was collected by centrifugation (2800rpm, 1200g, 5 minutes, 4°*C*). Post CM52 CETP (3mg) was added to the gel and mixed for 2 hours at 4°*C* on a Spiralmix (Denley). Gel was pelleted and the supernatant retained. The gel was washed two times with 10mM Tris HCl pH7.4 (3ml) and then eluted two times with 10mM Tris HCl, 20mM phosphate pH 7.4 containing 5mM dithiothreitol (3ml). Supernatant, washes and eluates were assayed for CETP activity.

For the Concanavalin A (con A) Sepharose purification of post CM52 CETP, a 5ml (0.8 x 10cm) con A Sepharose column was equilibrated with 50mM sodium acetate, 90mM NaCl pH 4.5 at 20 ml/hour. Post CM52 CETP (20ml) was loaded onto this column and the column washed with sodium acetate/NaCl buffer. The column was eluted first with buffer containing 10mM α-methyl-D-mannoside and then with buffer containing 150mM α-methyl-D-mannoside.

Anti-human albumin (Sigma, 1ml, 5.65mg) was coupled to CNBr activated Sepharose CL4B gel (1ml, 0.286g) as described previously for HDL. The column was washed four times with Tris-saline buffer and then post CM52 CETP (1mg) was loaded. The column was washed two times with Tris-saline buffer and then eluted with two times 1ml 0.2M glycine pH3.2. Washes and elution fractions were assayed for CETP activity.

For the chromatofocussing procedure, post CM52 CETP was dialysed against 25mM Tris HCl pH 7.4 and loaded in 15ml at 30 ml/hour onto a PBE-94 chromatofocussing gel (Pharmacia) equilibrated at room temperature in 25mM histidine base HCl pH 5.7 [Morton, R.E. & Zilversmit, D.B. (1982)]. This was followed by a cushion of 25mM histidine HCl (5ml). The column was eluted with 200ml Polybuffer 74 (Pharmacia, 25ml diluted to 200ml and adjusted to pH 3.5 with HCl). Fractions were collected and assayed for CETP activity.

2.3.2 Apolipoprotein AI Purification

Human HDL was prepared by ultracentrifugation as described previously. The HDL was then delipidated using ethanol:diethylether. Twenty-five ml extraction

solvent was required per 1ml HDL. All solvents were precooled to -20°C. Ethanol:diethylether (2:3 v/v) was added to HDL, mixed thoroughly and left to stand overnight at -20°C. As much solvent as possible was removed from above the white precipitate. Ethanol:diethyl ether (3:1 v/v) was added to the precipitate, mixed and left to stand overnight at -20°C. Again the solvent was removed, ethanol:diethyl ether 1:1 (v/v) added and the precipitate left to stand for 30 minutes. This last step was repeated using ethanol:diethylether (1:3 v/v). The protein precipitate was finally washed twice with diethylether and dried under a stream of nitrogen.

Delipidated protein (150mg) was dissolved in 0.1M Tris HCl pH 7.4, 0.01% EDTA, 5.4M urea (10ml) and loaded onto a Sephadex G-150 column (Pharmacia, 60 cm x 3cm) which had previously been equilibrated with the same buffer [Jackson, R.L. *et al* (1973)]. The column was eluted at 60ml/hour at room temperature with 1000ml buffer, collecting 10ml fractions. SDS PAGE was carried out on the fractions to detect those containing apoAI. Fractions containing apoAI, identified by the expected molecular weight of 28000, were pooled, dialysed against distilled water and then freeze-dried.

4 Immunological Techniques

2.4.1 Antibody Production

Secondary structure predictions were carried out by Mike Hann (Glaxo Group Research Ltd, Greenford). Chou & Fasman and Robson predictions were carried out with the aid of the computer program 'Predict' written by Peter Murray-Rust (Glaxo Group Research Ltd, Greenford). The eight secondary structure predictions used to give the consensus plot were the Burgess, Dufton, Fasman, Garnier, Kabat, Lim, McLauchlan and Nagano models using a computer program written by Elios Eliopolous (Leeds University).

The CETP peptide (residues 351-360, see Fig. 4.12) was synthesised by Dr Munns at Peptide and Protein Research, University of Reading. The peptide was coupled to keyhole limpet haemocyanin (KLH) via the N terminal cysteine according to the manufacturers instructions (CRB). KLH was dialysed against 10mM potassium phosphate pH 7.2 and then adjusted to a protein concentration of 20mg/ml. KLH 8mg (0.4ml of a 20mg/ml solution) was conjugated to 10mg peptide as follows. m-Maleimido benzoic acid N-hydroxysuccinimide ester (170µl of 3mg/ml, in dimethyl formamide) was added dropwise to 8mg KLH in 10mM potassium phosphate pH 7.2. The mixture was stirred for 30 minutes at room temperature. The activated KLH was desalted on a 20ml P30 column (Bio-Rad). The column was preequilibrated and run in 50mM potassium phosphate pH 6.0 at 10ml/hour. Fractions (1ml) were collected and the protein peak at fractions 13 to 16 was pooled. The activated KLH solution was added to a 5mg/ml peptide solution in 10mM potassium phosphate while stirring at room temperature. The pH was adjusted to 7.4 and the mixture was left to stir for 3 hours at room temperature.

KLH-peptide conjugate (2.6mg/ml) was mixed with an equal volume of Freund's complete adjuvant and sonicated at maximum amplitude to give a stable emulsion (6-10 seconds). Four subcutaneous injections of a preparation containing approximately 10μg peptide were given to a Black Face sheep; two injections were suprascapular and two were on the haunches. The sheep was boosted four weeks later with the same amount of antigen but using incomplete Freunds adjuvant. The sheep was bled two weeks after the boost. Immunisation was carried out by Peter Boulton (Glaxo Group Research Ltd, Greenford).

Mice were immunised with either post CM52 CETP (rabbit or human) or CETP bound to nitrocellulose prepared as follows. CETP bound to Intralipid was run overnight on a 10% SDS polyacrylamide gel loaded at approximately 35 to 135μg per well, and then blotted onto 0.2 micron nitrocellulose (as described later). Protein bands were stained with Ponceau Red and the band corresponding to the correct molecular weight for CETP was cut out and destained in PBS. Nitrocellulose was dissolved in a minimum volume of DMSO and excess DMSO was evaporated overnight under a stream of nitrogen in a 37°*C* water bath.

Mice were immunised with 1 to 10μg antigen by Paul Hissey (Glaxo Group Research Ltd, Greenford). The CETP preparations were diluted in PBS and mixed with an equal volume of incomplete Freunds adjuvant. This was sonicated at 10 microns amplitude for 30 seconds. Immunisation was by subcutaneous injection. Mice were tested and boosted at approximately four weekly intervals until serum samples from tail bleeds from the mice showed inhibition of CETP activity. Tail bleeds from mice immunised with human CETP were also tested by ELISA. Positive bleeds were achieved from the rabbit CETP immunisation in two months and from the human CETP immunisation in three months.

Monoclonal antibody production was carried out by Peter Boulton (Glaxo Group Research Ltd, Greenford). Mice were boosted with 10μg antigen before fusion. Mouse spleen cells were fused with NS1 cells (P3/NS1/1-Ag4-1) and cultured in Biocult DMEM containing 10% foetal calf serum. Cell supernatants were tested for their inhibition of a CETP assay, with the addition of screening by ELISA for the cells from the human CETP immunisation. Rabbit clones were tested for inhibition of both CE and TG transfer. Cultures were cloned three times to 0.3 cells per well. Wells were checked by eye under a microscope for growing clones. Seven days prior to innoculation of mice for antibody production in the ascitic fluid, mice were treated with Pristane (2,6,10,14, tetramethylpentadecane, 200μl) intraperitoneally. Mice were innoculated with a minimum of 10^6 cells per mouse. Ascitic fluid was tapped after 10 to 14 days and the cells removed by centrifugation. To purify the antibodies from the ascites, a 40% ammonium sulphate precipitation was carried out and then the precipitate was dissolved in PBS. This was then run on a Sephadex G200 column (Pharmacia) where the antibodies elute in the exclusion volume.

2.4.2 ELISA

A general method for ELISA is detailed here followed by descriptions of the particular applications used. An ELISA plate (Maxisorp, Nunc) was coated with antigen at 1μg/ml in 0.1M carbonate/bicarbonate buffer pH 9.6, 100μl per well. The plate was incubated with shaking for 1 hour at 37°C and washed three times with PBS 0.1% Tween 20. Plates were then blocked with 1% BSA in PBS 0.1% Tween 20 for 30 minutes at 37°C followed by three washes with PBS 0.1% Tween 20. The first antibody is added in PBS Tween, 100μl per well and incubated for 1.5 hours at 37°C. Again the plate was washed three times with PBS Tween. Second antibody, conjugated to horse radish peroxidase, usually 100μl of a 1/1000 dilution, was added in PBS Tween. The plate was incubated for 1.5 hours at 37°C. After washing three times with PBS Tween the plate was developed by adding 100μl of developing solution. Developing solution contained 100μl 10mg/ml tetramethyl benzidine, 100μl H_2O_2 (10μl 30% H_2O_2 in 670μl 0.1M citrate pH 6.0) in 10ml citrate buffer. The plate was then incubated for 15 minutes at room temperature and the reaction was stopped with the addition of 25μl of 2.5M H_2SO_4. The absorbance of the plate was read at 450nm and the background reading at 650nm was subtracted.

For testing for antibodies to the KLH-CETP peptide conjugate in sheep, an ELISA plate was coated with CETP peptide, KLH, human post CM52 CETP or rabbit post CM52 CETP at 1μg/ml. Sheep anti-KLH peptide serum was added in half-log dilutions starting at 1/300. Second antibody was donkey anti-sheep IgG peroxidase linked (Sigma) at a dilution of 1/1000.

For screening tail bleeds from mice immunised with human CETP, the tail bleed sera were added in doubling dilutions starting at 1/10. The second antibody used was goat anti-mouse IgG (Sigma) at a 1/1000 dilution.

When cell supernatants were tested for antibodies to human CETP, plates were coated with human post CM52 CETP at 50μg/ml (or 2μg/ml human serum albumin (HSA) for control). Plates were blocked with 0.5% Marvel and antibodies were added in PBS TWEEN containing 1% BSA. Supernatants were tested undiluted (or 1/3000 monoclonal anti-HSA (Sigma) was used as a control). The second antibody used was goat anti-mouse IgG at a dilution of 1/1000.

2.4.3 Western Blots

The basic method used for Western blotting is described below. Gels were blotted onto nitrocellulose (usually 0.2 micron) for 3 hours at 250mA in transfer buffer (25mM Tris pH 8.3, 192mM glycine, 20% methanol). After electroblotting, membranes were blocked for 30 minutes at 37°C in PBS, 0.05% Tween 20, 5% BSA. The membrane was then incubated with first antibody diluted in blocking buffer for 2 hours at room temperature. This was followed by one wash for 10 minutes in water and two washes for 10 minutes each in PBS, 0.05% Tween 20. The membrane was then incubated with second antibody for 1 hour at room temperature and then

washed once with water for 10 minutes, two times with PBS 0.05% Tween 20 for 10 minutes each and a further 10 minute wash in water. Substrate was then added for development (2mg/ml 4-chloro-1-napthol in methanol, 6.5ml, 30% H_2O_2, 12.5µl, and 18.75ml PBS). The reaction was allowed to develop for 10 to 40 minutes and was stopped by dilution in water.

For the blot to check the identity of the CETP protein using an anti-human CETP mouse IgM (Hans Dieplinger, Innsbruck), TBS (20mM Tris, 500mM NaCl pH7.5) was used instead of PBS. The first antibody dilution was 1/50. The second antibody was goat anti-mouse IgM conjugated to horse radish peroxidase (Sigma) at a dilution of 1/500.

For the blots using sheep antiserum the membrane was washed in 0.01% Tween 20, 0.025% Sarkosyl in PBS. The menbranes were blocked in 5% BSA and 0.5% Marvel. The first antibody was a 1/100 dilution of sheep antiserum and the second conjugated antibody was 1/1000 donkey anti-sheep IgG.

For the blot using antibodies from the mouse ascitic fluid, a 40% ammonium sulphate precipitate of mouse ascites was used. The Western blot used 1/50 dilution of the mouse antibody preparation and 1/500 dilution of goat anti-mouse IgG was used as the conjugated second antibody.

5 Animal Diets

2.5.1 Rabbits

Froxfield rabbits were obtained from Froxfield, Southampton. These rabbits have a deficiency in their LDL receptor inherited in a Mendelian manner, transmitted as an autosomal dominant characteristic. These rabbits have high serum cholesterol and spontaneous atheroma. The animals were 8 to 12 weeks of age at the start of experiments. Only male homozygotes were used. When rabbits were allocated to control and treatment groups they were divided such that each group had similar mean cholesterol. For the probucol experiment Froxfield rabbits were fed 1% probucol incorporated into FD1 diet for 12 weeks. The ACAT inhibitor was administered orally as an emulsion in 5% corn oil and 1% Tween 80. The final concentration fed was 3% GR69918X. The antioxidant GR44966X was dissolved in chloroform, sprayed over FD1 diet and the chloroform allowed to evaporate. Salbutamol was incorporated at 0.03% into the FD1 diet and fed to rabbits for 12 weeks. All animal diets were obtained from Special Diet Services Ltd (SDS).

Dutch Belted Rabbits were used for the fat feeding experiment. Control diet was FD1 and the treatment was high fat and low cholesterol (0.1%) SDS diet. Dutch Belted rabbits were used at 12 weeks of age and fed the diet for 11 weeks. All rabbit blood samples were taken from the marginal ear vein.

2.5.2 Marmosets

Marmosets were fed according to the following regime:-
Monday - AM dry pellets and 1/6 slice bread; PM 1/8 orange, apple, 2 slices of banana. Tuesday - AM pellet mash and 1/6 slice bread; PM treat. Wednesday - AM dry pellets and 1/6 slice bread; PM 1/8 orange, apple, 2 slices banana. Thursday - pellet mash and 1/6 slice bread; PM treat. Friday - AM dry pellet and 1/6 slice bread; PM 1/8 orange, apple, 2 slices banana. Saturday - pellet mash, 1/6 slice bread and treat. Sunday - pellet mash, 1/6 slice bread and 1/8 apple. All fruits were washed prior to feeding. Pellet mash - per marmoset = 20g Marzuri marmoset pellets mixed with 1 pint of UHT milk and Glucodin. Bread = wholemeal bread. Fresh water was provided daily. Treats included grapes, marmoset jelly, monkey nuts and sugar puffs.

Marmosets were dosed intragasrically with GR92549 dissolved in water for injection. Blood samples were taken from the tail vein.

6 Cell Work

2.6.1 Cell Culture

CaCo2 cells were cultured in Transwells (Costar) in MEM (with Hank's salts), 10% foetal calf serum, 1% non essential amino acids, 1% glutamine, 0.1% penicillin and 0.1% streptomycin. Transwells were incubated overnight with medium before use. CaCo2 cells were harvested by trypsinisation and resuspended at a concentration of $1x10^6$ cells per ml. Cells (2ml) were put in the top compartment and medium (2ml) in the bottom. Cells were used at 15 days post confluence.

HepG2 cells were cultured in MEM, Hank's salts with 8.8mM glycine. Medium was supplemented with 1% glutamine, 0.1% penicillin, 0.1% streptomycin, 1% non essential amino acids and 10% heat inactivated foetal calf serum [Faust, R.A. & Albers, J.J. (1987)]. Cells were harvested by trypsinisation and subcultured every five days.

J774 cells were cultured in RPMI 1640 medium containing 1% glutamine, 0.1% penicillin, 0.1% streptomycin and 10% foetal calf serum. Cells were harvested by scraping and subcultured every three days at a density of 0.5 x 10^6 cells/ml.

To Trypan blue stain cells, Trypan blue (50µl of a 5mg/ml solution of Trypan blue (BDH) in PBS stored after filtering) was added to a cell sample (50µl) and mixed. Cells were viewed on a haemocytometer under a microscope and total cells and blue stained (dead cells) were counted.

2.6.2 Isolation of Human Monocyte-Derived Macrophages

Blood (250ml) from one donor was collected into 5 x 50ml tubes, three of which contained 50mg solid EDTA as anticoagulant and two of which contained no anti-coagulant. The tubes with EDTA were mixed on a Spiralmix (Denley) for 5 minutes.

The two tubes without anticoagulant were used to prepare serum which was then filter sterilised through a 0.2 micron filter. Subsequent stages were carried out in a laminar flow cabinet. The whole blood from the three tubes containing EDTA was transferred into plastic universals (10ml per tube) and underlayed with an equal volume of Ficol-Paque (Pharmacia) using a syringe and a 19 gauge needle [Fogelman, A.M. *et al* (1979)]. The tubes were centrifuged at 4°C (1600rpm, 400g, 30 minutes). The top layer of plasma (4ml) was removed to just above the interface. The cells at the interface were removed in 2-3ml, using a syringe and needle, and pooled. The cells were diluted 1 to 1 in PBS and centrifuged (16000rpm, 400g, 20 minutes). The cell pellet was washed two times with PBS and suspended in 20ml RPMI 1640 medium supplemented with 0.1% penicillin and 0.1% streptomycin. The cell suspension was aliquotted into flasks precoated with 0.1mg/ml poly-d-lysine. Usually cells from 150ml blood were put into one large (175 cm^2) flask or two medium (75cm^2) flasks or 40 wells from six well plates. The flasks were incubated overnight at 37°C. Non-adherent cells were then poured off. The adherent monocytes (some platelets remain also) were cultured in RPMI 1640, 0.1% penicillin, 0.1% streptomycin, 1% glutamine, 20% autologous human serum for 7 to 15 days before use.

2.6.3 Oleate Albumin Preparation

Oleic acid (1g) was washed into a beaker with a minimum volume of ethanol. Two drops of phenolthalein (0.1g/250ml in 70% ethanol) was added. The acid was neutralised with 1M NaOH until the solution was just pink. The ethanol was then evaporated under nitrogen. PBS (50ml) was added and the mixture boiled. The final concentration was adjusted to 20mg/ml oleic acid. Equal volumes of 20mg/ml oleate mix and 20% BSA in PBS were mixed and filter sterilised. The final concentration was 10mg/ml (32.5mM) oleate 10% BSA [Patsch, W., Tamai, T. & Schonfield, G. (1983)].

2.6.4 Preparation of Acetyl LDL

LDL (15mg protein in 10ml) was concentrated to 2ml by placing in dialysis tubing and covering it with sucrose crystals (about 1 hour). Concentrated LDL was dialysed overnight against 1L of 0.9% saline at 4°C. LDL was then added dropwise with stirring to an equal volume of cold (4°C) saturated sodium acetate solution [Basu, S.K. *et al* (1976)]. After stirring for 10 minutes on ice, acetic anhydride with a total mass equal to five times the weight of LDL protein present was added in 0.5µl aliquots over 1 hour. For 15mg protein, 0.5µl was added every 15 seconds for 10 minutes and every 30 seconds for 50 minutes (total of 70µl). The mixture was left to stir on ice for a further 30 minutes before overnight dialysis against 1L PBS at 4°C.

7 Molecular Biology Methods

2.7.1 Purification of DNA and RNA

DNA was prepared according to the method of Kunkel *et al* [Kunkel, L.M. *et al* (1977)]. Cells from 10ml blood were mixed with 0.32M sucrose, 10mM Tris HCl pH 7.5, 5mM MgCl, 1% Triton X-100 (80ml) at 4°*C*. Nuclei were collected by centrifugation (10000rpm, 10 minutes, 4 °*C*). The pellet was suspended in 0.075M NaCl, 0.024M EDTA pH 8.0 (4.5ml) using a pasteur pipette. 10% SDS (250µl) and freshly made proteinase K solution in water (10mg/ml, 50µl) were added and the resuspended nuclei incubated overnight at 37°*C*. Water saturated phenol (5ml) was added and the tube then centrifuged (10000rpm, 5 minutes, room temperature) and the upper phase removed. Chloroform:isoamylalcohol (24:1, 5ml) was added, the tube mixed gently and centrifuged. The upper phase was removed and re-extracted with chloroform:isoamylalcohol. Sodium acetate (3M, pH 5, 0.5ml) and 100% ethanol (11ml) were added to the upper phase at room temperature. The tube was inverted several times and the DNA removed with a pasteur pipette. DNA was dissolved in 10mM Tris pH 8.0, 1mM EDTA (1ml) at 4°*C* and the concentration was determined by absorbance at 260nm.

For RNA isolation from cells in culture one large (175cm^2) tissue culture flask (10-40 x 10^6 cells) was lysed with 2.5ml 4M guanidium isothiocyanate, 5mM sodium citrate pH7.0, 0.1M β-mercaptoethanol, 0.5% Sarkosyl [Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982)]. If tissue was used then 1g was homogenised in 2.5ml of the guanidium isothiocyanate solution. Caesium chloride (1g) was added to each 2.5ml of dispersed tissue or cells. The homogenate was layered onto a 1.2ml cushion of 5.7M CsCl in 0.1M EDTA pH 7.5 in a Beckman SW50.1 polyallomer tube and overlaid with mineral oil. The tubes were centrifuged (35000rpm, 65 hours, 20°*C*) using slow acceleration and deceleration profiles in a SW50.1 rotor in a Beckman L8-70M ultracentrifuge. The supernatant was discarded and the tube inverted on a paper towel. The RNA pellet was dissolved in 10mM Tris HCl pH 7.4, 5mM EDTA, 1% SDS (300µl). This was transferred to a microfuge tube and extracted with chloroform:1-butanol 4:1 (300µl). The phases were separated in a microfuge (13000rpm, 5 minutes). The upper aqueous phase was removed and the organic phase re-extracted with 10mM Tris HCl pH 7.4, 5mM EDTA, 1% SDS (300µl). The two aqueous phases were combined. A one tenth volume of 3M sodium acetate pH 5.2 and 2.2 volumes of ethanol were added and the mixture stored overnight at -20°*C*. The RNA was recovered by centrifugation in a microfuge (13000rpm, 15 minutes). The RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water and the RNA concentration determined spectrophotometrically. mRNA was purified on oligo (dT)-cellulose using the Pharmacia mRNA Purification Kit as described by the manufacturer.

2.7.2 Gel Electrophoresis of RNA and DNA

A 5μg sample of DNA was digested overnight with restriction enzymes according to the manufacturers' instructions in the presence of 4mM spermidine. The digested DNA was electrophoresed through 0.8% agarose gels (10.5 x 13cm) in 1 x TAE buffer (50 x TAE = 0.04M Tris, 57.1ml/l glacial acetic acid, Mm K_2EDTA pH 8.0) overnight at 40mA alongside markers from lambda DNA cut with HindIII. After electrophoresis gels were stained in ethidium bromide (2.5μg/ml, 5 minutes) and photographed.

For RNA gels samples were dried down in a Savant vacuum drier and then DEPC treated water (11μl), formamide (25μl), formaldehyde 37-40% (9μl) and 15 x MOPS (5μl, 5 x MOPS = 0.2M morpholinopropane sulfonic acid pH 7.0, 50mM sodium acetate, 5mM EDTA pH 8.0) were added. Tubes were heated at 55°*C* for 15 minutes. Sample buffer was added (3μl, 6 x sample buffer = 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) and the samples were electrophoresed in a 1.2% agarose gels (20 x 25cm) containing 1 x MOPS buffer and 2.2M final concentration formaldehyde [Maniatis, T, *et al* (1982)]. Formamide and formaldehyde were deionised before use by stirring for 1 hour with 5g/100ml AG 501 X8 (D) ion exchange beads (Bio-Rad). Gels were run overnight at 60V with the buffer tanks stirred. Gels were stained in 0.5μg/ml ethidium bromide, 30 minutes and then destained 3 x 1 hour in 3 x SSC (20 x SSC = 0.3M sodium citrate, pH7.0, 3M NaCl).

2.7.3 Blotting

DNA was transferred to Hybond-N (Amersham) by Southern blotting [Maniatis, T. *et al* (1982)] and the hybond-N was UV irradiated for 3 minutes to bind DNA. RNA gels were soaked in 50mM NaOH, 10mM NaCl (45 minutes, room temperature), neutralised in 0.1M Tris HCl pH 7.5 (45 minutes) and then soaked in 20 x SSC before Southern blotting.

Slot blots were carried out using a Schleicher and Schuell Minifold II slot blot apparatus. Filter paper and Hybond-N were presoaked in 6 x SSC. RNA samples were prepared in 2.2M formaldehyde, 50% formamide in DEPC-treated water and heated at 65°*C* for 15 minutes, whereas DNA samples were loaded in 0.2M NaOH. Samples (500μl) were loaded over about 1 hour. Membranes were UV irradiated for 3 minutes and baked in an oven for 2 hours at 80°*C*.

2.7.4 Probe Labelling

Three probes were used for hybridisation as described in Chapter VI. The 1581bp cDNA probe comprising the coding sequence for amino acid 8 to 476 of the protein [Drayna, D. *et al* (1987)] was prepared from the plasmid CETP-II which was a gift from Dr D. Drayna (Genetech Inc. CA). This was prepared by digestion of the plasmid with EcoRI and cutting the appropriate fragment out of a low melting point gel. Subfragments, 732bp and 362bp, of this probe were prepared as described in Chapter VI by Dr D. Gaffney (Glasgow Royal Infirmary). The 75 bp oligonucleotide

probe GR91788X was synthesised by Dr Barry Coomber (Glaxo Group Research Ltd, Greenford). The actin probe obtained from Oncor (cat. no. P7000) was a 770bp probe from the chicken sequence. A further probe was used for apoAI RFLP analysis. This was a 2.2kb PstI genomic DNA fragment of apoAI and was a gift from Dr S. Humphries (Charing Cross Sunley Research Centre, Hammersmith, London). The hybridisation conditions used for this probe were identical to those used for the 1581bp CETP cDNA probe. Hybridisation conditions for these probes are detailed in Table 6.4.

CETP cDNA and apoAI probes were labelled using the random priming method [Feinberg, A.P. & Fogelstein, B. (1984)] and purified on a G50 (Pharmacia) column in 10mM Tris pH 8.0, 1mM EDTA, 0.1% SDS. These two probes were labelled to specific activity 4-9 x 10^5 cpm/ng and added to a minimal volume of hybridisation mixture [Maniatis, T. *et al* (1982)] at a radioactive concentration of 2.5-5 x 10^6 cpm per blot. Probes prepared in this way were used for hybridisation to DNA blots as described in Chapter VII. Actin and latterly the CETP cDNA probe were labelled using the Multiprime DNA Labelling System Kit (Amersham) as described in the manufacturer's instructions. For labelling GR91788X, components of this kit were used but the Primer Solution was replaced with a synthetic primer GR91789X (synthesised by Dr B. Coomber, Glaxo Group Research Ltd, Greenford) in an equimolar amount to GR91788X (i.e. 5.5 pmol/250µl labelling preparation) and nuclease-free BSA at a final concentration of 200µg/ml. Briefly, 125ng probe was denatured by boiling for 2 minutes and placed on ice. Amersham Buffer Solution 1 (50µl) and Primer Solution 2 (25µl) or GR91788X plus BSA were added and the volume made up to 115µl with DEPC water. ^{32}P-dCTP (25µl, 3000Ci/µmol, 250µCi) was added and then Amersham Solution 3 (DNA polymerase I Klenow fragment, 10µl). The solutions were mixed by pipetting and incubated at room temperature for 5 hours. Sodium acetate 3M (25µl) and sheared herring sperm DNA (167µl, 10mg/ml) were added and the tube mixed. The probe was left to stand overnight at -20°*C* to precipitate. Probe was recovered by centrifugation (13000rpm, 10 minutes). The supernatant was removed and the pellet dissolved in DEPC-treated water (500µl) by boiling and mixing with a pipette. If necessary, the labelled probe preparation could be stored at -20°*C*. Typical specific activities of these probe are shown in Table 6.3. Probes prepared in this way were used for hybridisation to RNA blots as described in Chapter VI.

2.7.5 Hybridisation and Washing Blots

For DNA blots, gels were prehybridised in 5 ml hybridisation mix (6 x SSC, 5 x Denharts solution, 0.5% SDS, 20µg/ml denatured salmon sperm DNA) [Maniatis, T. *et al* (1982)] for four hours and then hybridised in the same solution containing denatured radiolabelled probe. After hybridisation, blots were washed in 2 x SSC,

0.5% SDS at room temperature for 10 minutes, 2 x SSC, 0.1% SDS at room temperature for 15 minutes, 2 x SSC, 0.5% SDS at 65°C for 1 hour and finally in 2 x SSC, 0.5% SDS at 65°C for 30 minutes.

For the RNA blots, hybridisation conditions were as described in Table 6.4. Blots were prehybridised in hybridisation fluid with or without formamide (Hybrisol I or II respectively, Oncor) at the appropriate temperature overnight. Hybridisation was carried out overnight in 25ml Hybrisol containing 125ng labelled probe. Blots were then washed in 1L 2 x SSC at room temperature, 1L 2 x SSC, 0.1% SDS at room temperature and 1L 0.1 x SSC at 35ml/hour in a automated washing system (Hybaid). Blots were autoradiographed for 1 to 7 days.

2.7.6 Polymerase Chain Reaction (PCR)

DNA (0.1pg-1ng) was incubated with 10µl of 10 x concentrated PARRS Excellence buffer (Cambio), 5ng/ml final concentration of sense and antisense primers (British Biotechnology Ltd, primers shown in Fig 6.11, 10µl of 50ng/µl solution of each), and deoxyribonucleotides (dNTPs 16µl of stock solution containing 1.25mM of each dNTP) [Erlich, H.A. (1989)]. The volume was made to 100µl with DEPC treated water. The mixture was overlaid with mineral oil (100µl) and heated for 6 minutes at 92°C and then cooled on ice. Taq polymerase (Cambio, 5U, 1µl) was added and the tube put through 30 heating cycles. One cycle comprised heating at 92°C for 1 minute, 50°C for 1 minute and 70°C for 2 minutes.

PCR reaction mix (10µl) was mixed with sample buffer (5µl, 1.25% SDS, 2.5M EDTA, 15% Ficoll, 0.15% bromophenol blue). This was loaded onto a 2% agarose horizontal gel containing 0.5µg/ml ethidium bromide. A 123bp DNA ladder (Pharmacia) and a track of DNA sample buffer were also run on the gel. The gel (11 x 13,5cm) was electrophoresed for 3 hours at 70 V, using Tris borate running buffer (5 x TBE = 445mM Tris base, 445mM boric acid, 10mM EDTA, Sigma). The gel was then photographed under UV illumination.

8 Population for RFLP analysis

A group of 56 subjects (34 men and 22 women) aged 20 to 59 were selected from an opportunistic screening programme in Glasgow on the basis of their HDL levels. Normotryglyceridaemic (<3.0mM) individuals with low HDL levels (less than 1mM) or high HDL levels (greater than 2.5mM for females and greater than 2.0mM for males) were recalled after the initial screening visit and measurements made of total cholesterol, HDL cholesterol, CETP activity and LCAT activity from fasting samples. Blood (10ml) was collected into potassium EDTA tubes and separated into plasma and cells by centrifugation. Both fractions were frozen at -20°C. The cell fraction was used for leukocyte DNA extraction as described in section 2.7.1. and the plasma was used to prepare lipoprotein deficient plasma for CETP and LCAT determinations.

CHAPTER III

DEVELOPMENT OF A CHOLESTERYL ESTER TRANSFER PROTEIN ASSAY

1 Introduction

The assay of CETP activity is complex and its activity can be measured in different ways. CETP can be assayed either as an exchange activity or as a net transfer activity. CE and TG can be transferred in both directions between lipoprotein substrates and this exchange can be measured. A radiolabelled assay measures the rate of exchange of lipids by measuring accummulation of radiolabel in an acceptor lipoprotein after transfer from a labelled donor particle. It does not measure the absolute amount of lipid transferred because any CE or TG in the acceptor particles is free to be transferred back to the donor particle by the same exchange activity. A mass transfer assay measures the actual mass of CE and TG that is transferred from one lipoprotein to another. This therefore measures the net result of forward and back transfer. An exchange reaction does not necessarily alter lipoprotein composition whereas net transfer does due to unidirectional transfer of lipids.

An exchange assay using radiolabel is quick with easily detectable amounts of exchange. This assay still provides a measure of the amount of enzyme present. A mass transfer assay requires accurate measurement of small changes in lipid but provides information on a particular aspect of CETP activity [Ogawa, Y. & Fielding, C.J. (1985)].

When measuring CETP activity in plasma samples, other factors may influence the level of activity measured. Endogenous lipoproteins may affect transfer rates since the rate of exchange is affected by the concentration and composition of substrate lipoproteins. In the assay used here, CETP activity was separated from the endogenous lipoproteins by ultracentrifugation. Some CETP may remain in the HDL fraction when LPDP is separated and hence it is important to compare activities between samples prepared in the same way. Activity was measured in the LPDP using standardised amounts of exogenous lipoprotein substrates. Thus the maximum amount of exchange that occurs when optimum substrate concentrations are chosen is determined.

Since LPDP samples also contain CETP inhibitor protein [Tollefson, J.H. *et al* (1988), Nishide, T. *et al* (1989)], therefore any activity measured will be the net result of CETP plus inhibitor protein. This will not give an indication of how much CETP protein is present but does measure the amount of CETP activity that is normally

expressed in a particular LPDP sample. It is not known how inhibitor is distributed *in vivo*. It has been purified from HDL and seems to bind HDL as does CETP. It is yet to be ascertained whether inhibitor is removed from HDL by ultracentrifugation like CETP and also which is the more physiologically relevant fraction of each of these proteins. Other plasma enzyme activities may affect CETP. Lipoprotein lipase has been shown to stimulate CETP activity and LCAT can produce CE from cholesterol and may affect the specific activity of the CE in the assay.

It was decided to develop an assay which measured exchange activity. This type of assay enabled handling of large numbers of samples more easily and would still give an indication of relative amounts of transfer activity in different samples when standard donor and acceptor particles were used. The assay developed was based largely on a method previously reported by Tollefson and Albers [Tollefson, J.H. & Albers, J.J. (1986)]. The principle of the assay is to measure accumulation of radiolabelled CE in an acceptor particle after transfer from a labelled donor. Donor lipoproteins are labelled by incubation of lipoprotein and a lipid emulsion containing radiolabelled CE (or TG) with a CETP source. The CETP transfers labelled lipid into the lipoprotein particle and the lipoproteins are reisolated. To assay CETP activity, labelled donor lipoprotein is incubated with a non-labelled acceptor lipoprotein in the presence of CETP. At the end of the incubation the lipoproteins are separated and the loss of label from the donor lipoprotein is determined. In the technique described here the lipoproteins are separated by heparin manganese precipitation which relies on the precipitation of the larger apoB containing lipoproteins. Thus the LDL and VLDL are precipitated leaving HDL behind in the supernatant. HDL_3, a subfraction of HDL, was used since it contains no apoE which can interact with heparin and possibly precipitate. Transfer was calculated as the amount of radioactivity lost from the donor particle as a percentage of the total radioactivity, and expressed as percent transfer per volume of sample or per μg sample protein. Various adaptations were used which improved the assay in our hands. These changes included using a different technique for labelling lipoprotein donors [Morton, R.E. & Zilversmit, D.B. (b) (1981)] and adapting assay volumes to suit our needs.

2 Preparation of Substrates

3.2.1 Radioactive Labelling of Donor Particles

The labelled donor particle used for the assay was an HDL fraction labelled with radioactive cholesteryl oleate. This was prepared according to the method of Morton and Zilversmit [Morton, R.E. & Zilversmit, D.B. (b) (1981)]. The technique used the endogenous CETP activity in a plasma sample to label the endogenous lipoproteins in the plasma using a synthetic lipid emulsion as a donor. This process is described in detail in the Methods.

Using whole plasma in the labelling procedure, the VLDL, LDL and HDL lipoprotein fractions were labelled to different extents (Table 3.1). The labelled lipoprotein fraction required for the CETP assay was a subfraction of HDL, HDL_3. In an attempt to make labelling of the required HDL fraction more efficient, the unwanted lipoprotein fractions were removed from plasma by ultracentrifugation prior to incubating the sample with the labelled synthetic emulsion. This resulted in increased incorporations and specific activities of labelled substrates (Table 3.2). It is possible to label lipoproteins with [^{3}H] or [^{14}C] labelled either in the CE or TG moiety. Table 3.2 compares the specific activity of, and percentage incorporation into, different labelled HDL preparations. Tritiated cholesteryl oleate was used in preference to ^{14}C labelled cholesteryl oleate since it was supplied at a higher specific activity resulting in more incorporation of radioactivity into the substrate and thus allowing for more accurate determination of transfer of labelled lipid. When labelled lipoproteins were run on a Lipofilm gel, stained for lipid and protein and then autoradiographed, it can be seen (Fig 3.1) for the labelled LDL and HDL fractions that the radioactivity is coincident with the LDL and HDL bands visualised by the stains. Routinely ^{3}H-CE HDL_3 was used as this HDL fraction was not appreciably precipitated by heparin manganese, showed high specific activity and had good incorporation of label.

3.2.2 Removal of Contaminating Transfer Activity From Substrates.

An early problem encountered was the detection of substantial exchange activity in the absence of any added CETP source i.e. the blank transfer rate was too high. Rates as high as 30% transfer over the duration of the assay incubation could be detected - much too high for an assay which is only linear to about 30% transfer.

At first it was thought that this phenomenon may have been due to release of labelled lipid from the substrate into the assay medium due to instability of the prepared substrate especially at the higher assay temperature of 42°*C*. In order to stabilise the assay components the assay was carried out in the presence and absence of bovine serum albumin (BSA, 3% w/v). As can be seen from Fig. 3.2 blank rate transfer over 6 hours at 42°*C* in the absence of BSA was 25%. This was little improved by the inclusion of 3% BSA in the assay (16% transfer).

Another possible explanation for the high blank transfer rate was that CETP from plasma remained bound to either of the lipoprotein substrates during their isolation and could therefore stimulate lipid exchange in the assay. There are reports that CETP is found bound to lipoprotein fractions especially HDL [Groener, J.E.M. *et al* (1984), Pattnaik, N.M. & Zilversmit, D.B. (1979)].

LIPOPROTEIN FRACTION	% TOTAL ^{14}C-CE RECOVERY	SPECIFIC ACTIVITY OF FRACTION (dpm/μg protein)
VLDL	3	3315
LDL	30	1720
HDL	20	396

TABLE 3.1 **RECOVERY OF RADIOACTIVITY IN LIPOPROTEIN FRACTIONS FROM A LIPOPROTEIN LABELLING PREPARATION.** ^{14}C-cholesteryl oleate (20μCi) was used to label whole plasma (25ml) as described in the methods. After an 18 hour incubation with lipid emulsion, the lipoprotein fractions VLDL, LDL and HDL were harvested by ultracentrifugation at d=1.0063g/ml, d=1.063g/ml and d=1.21g/ml respectively after removal of chylomicrons. The percentage recovery of ^{14}C-CE was calculated as the amount of radioactivity recovered in each lipoprotein fraction as a percentage of the total used to label them. Specific activity of each fraction was calculated as the amount of radioactivity per microgram protein.

	n	SPECIFIC ACTIVITY (dpm/μg protein)	INCORPORATION (%)
^{3}H-CE HDL_{TOTAL}	2	1958	
^{3}H-CE HDL_3	21	9724 +/- 5054	19.5 +/- 8.7
^{14}C-CE HDL_3	2	327	20
^{14}C-TG HDL_3	2	1696	14
^{3}H-CE,^{14}-TG HDL_3	2	3230 2072	24 19

TABLE 3.2 **SPECIFIC ACTIVITIES OF LABELLED HDL PREPARATIONS.** Total HDL, using d>1.063g/ml plasma (20ml), or HDL_3, using d>1.125g/ml plasma (20ml), were labelled with either ^{3}H-CO (160μCi), ^{14}C-CO (20μCi), ^{14}C-triolein (TO) (20μCi) or ^{3}H-CO (160μCi) and ^{14}C-TO (20μCi) together. Specific activities were calculated as amount of radioactivity per microgram HDL protein. Incorporation was calculated as amount of radioactivity in HDL fraction as a percentage of that used to label it.

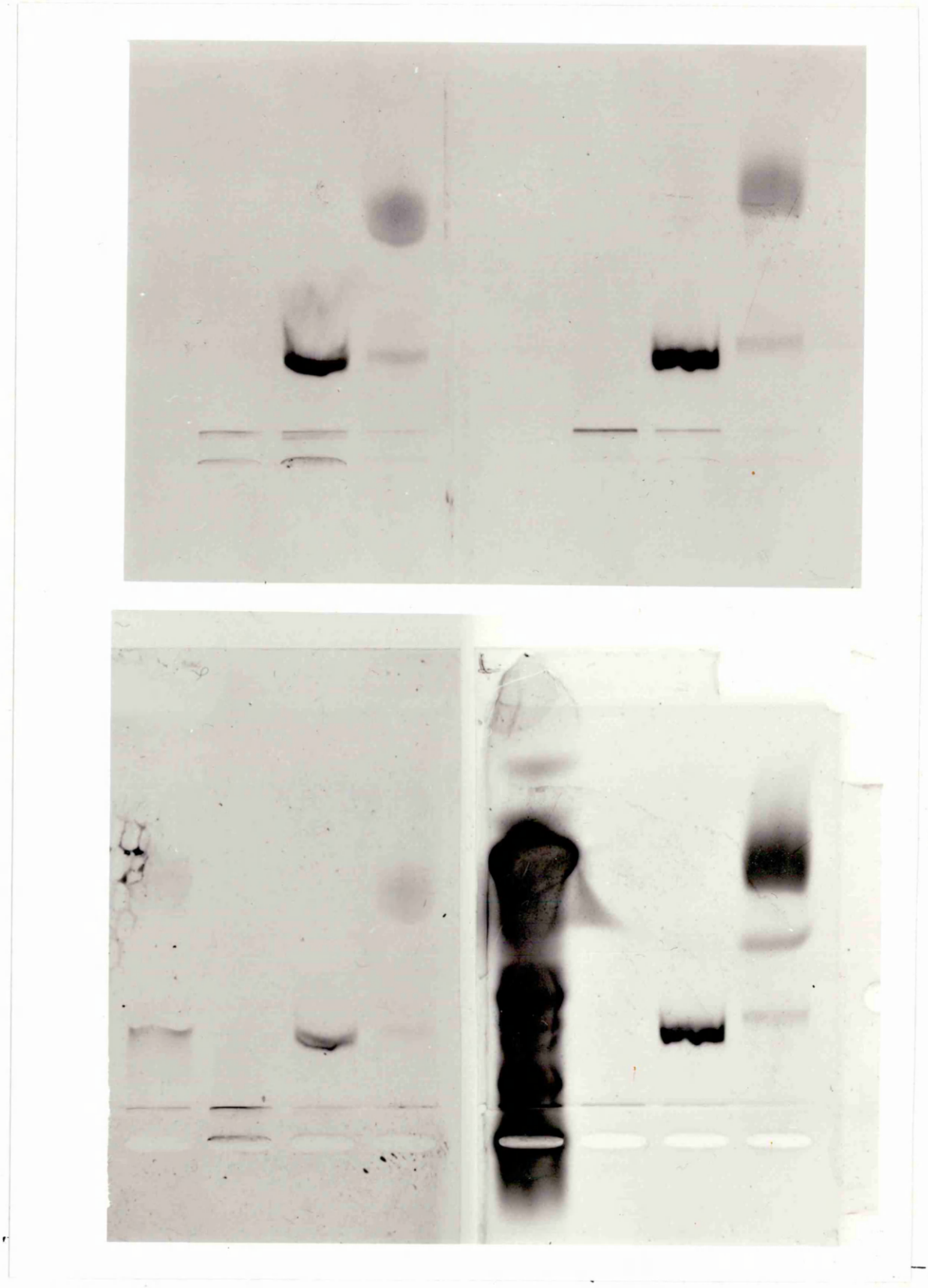


FIGURE 3.1 LIPOFILM AND AUTORADIOGRAPH OF LABELLED LIPOPROTEINS. Samples (5μl) of plasma (P), ^{14}C-VLDL (V), ^{14}C-LDL (L) and ^{14}-HDL (H) were run on a Lipofilm gel in duplicate sets. One half of the gel was stained with Sudan black (1) and the other with Coomassie blue (2). After staining the Lipofilm gel was autoradiographed for one week at -20°C (3).

Fig 3.2

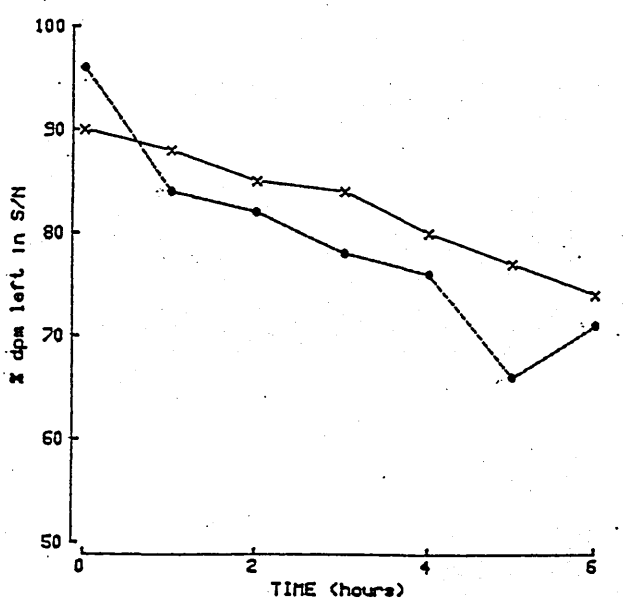


Fig 3.3

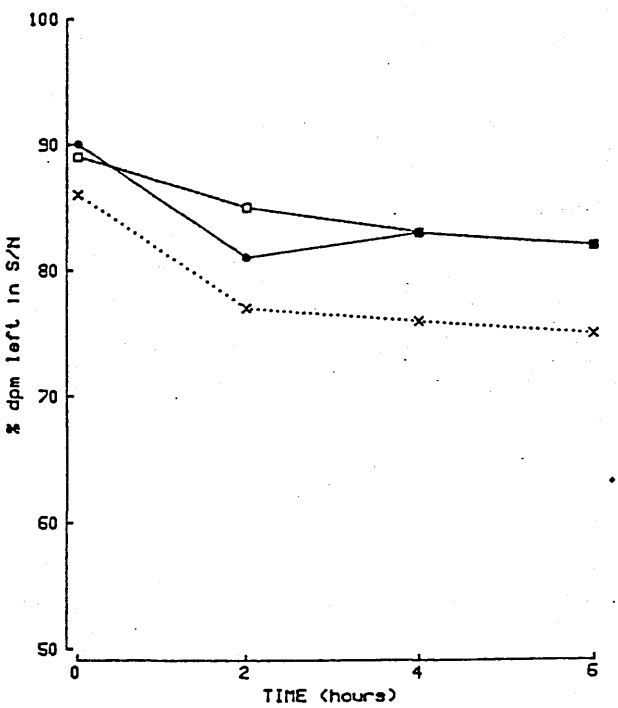


FIGURE 3.2 **CETP BLANK TIME COURSE AT 42°C - EFFECT OF BSA IN BUFFER.** ^{3}H-HDL$_3$ (5μg) was incubated at 42°C for varying lengths of time with cold LDL/VLDL (8μg)in the presence of Tris-saline buffer (●) or Tris-saline buffer containing 3% BSA final concentration (×). At the end of the incubation the lower density lipoproteins were separated by precipitation with heparin manganese reagent and the tritium left in the supernatant was determined. Counts remaining in the supernatant were expressed as a percentage of those added to the incubation.

FIGURE 3.3 **CETP BLANK TIME COURSE AT 42°C USING TREATED SUBSTRATES.** ^{3}H-HDL$_3$ (5μg) and LDL/VLDL (8μg) were incubated together for up to 6 hours at 42°C. At the end of the incubation high and lower density lipoproteins were separated by heparin manganese precipitation. ^{3}H-HDL$_3$ was counted in the supernatant. (●) - HDL$_3$ was subjected to ultracentrifugation at d=1.125g/ml followed by ultracentrifugation at d=1.215g/ml and LDL/VLDL was subjected to an extra centrifugation at d=1.063g/ml. (□) - HDL$_3$ was centrifuged, LDL/VLDL was untreated. (×) - LDL/VLDL was subjected to centrifugation, HDL$_3$ was untreated.

A possible way of removing this contaminating CETP was to subject the lipoprotein substrates to a further ultracentrifugation at their final density (see methods for details) to remove other contaminating lipoprotein fractions and to dissociate the CETP lipoprotein interactions [Groener, J.E.M. *et al* (1984)]. Fig. 3.3 shows the effect of centrifuging either the donor HDL or the acceptor LDL/VLDL or both to remove the contaminants. Assays where only LDL/VLDL was treated still showed a high blank rate of 11%. Assays where either HDL alone was treated or both HDL and LDL/VLDL were treated showed a much improved blank rate of 7% transfer. It was decided to treat both HDL and LDL/VLDL substrates by ultracentrifugation before use in the assay.

Ideally the blank rate should be as low as possible, therefore a further approach was used to separate any CETP from HDL particles, Since there is a large size difference between the CETP molecule (74K) and an HDL particle (200-400K), these two entities may be separable by gel filtration. After harvesting the labelled HDL_3 fraction from the second d=1.215g/ml density ultracentrifugation step, the lipoprotein was passed down a 100ml Sepharose CL-4B gel filtration column. A typical column profile is shown in Fig. 3.4. The HDL peak eluted at 70mls. When labelled HDL_3 is mixed with cold HDL_3 the peaks are coincident showing that at least with regard to behaviour on a gel filtration column, the labelling process does not alter the HDL_3 particle (Fig. 3.4). When double labelled HDL_3, labelled with ^3H-cholesteryl oleate and ^{14}C-triolein, is passed down the CL-4B column the ^3H and ^{14}C peaks are also coincident (Fig. 3.5). Experiments where CETP is passed down such a CL-4B column on its own (see Fig. 4.3A) show that free CETP would elute just after the HDL_3 peak at fraction 30 (79ml).

Fig 3.6 compares the effects of these various treatments for the removal of contaminating CETP activity on the blank transfer rates. If the HDL_3 is cleaned by gel filtration only the blank transfer rate is high (19% transfer). Cleaning by double ultracentrifugation improved this. However if the HDL_3 was cleaned by ultracentrifugation and then passed through a gel filtration column then the transfer rate was only 6% for at least 4 hours of the assay. Thus for all CETP assays the HDL_3 substrate was subjected to two ultracentrifugation steps, a gel filtration step and tested in a blank time course to check the blank rate.

3 Assay Incubation

3.3.1 Separation of Donor and Acceptor Particles

In order to measure the amount of labelled lipid transferred from the labelled donor lipoprotein particles to the unlabelled acceptor lipoprotein particles, these two entities must be separated efficiently at the end of the experiment. There are two main ways of doing this; separation by ultracentrifugation at d=1.063g/ml to remove the lower density lipoproteins, and precipitation by a technique which will discriminate between the donor and acceptor particles. Of the two techniques, precipitation lends

FIGURE 3.4 **SEPHAROSE CL-4B GEL FILTRATION COLUMN - CHROMATOGRAPHY OF LABELLED AND UNLABELLED HDL_3.** ^3H-HDL_3 (2mls, 0.25mg/ml, 2500dpm/μl) was mixed with HDL_3 (2ml, 6mg/ml) and loaded onto a 100ml Sepharose CL-4B gel filtration column. Protein was eluted at 11ml/hour with Tris-saline buffer. fractions (2.75ml) were collected and samples were counted by liquid scintillation counting.

FIGURE 3.5 **SEPHAROSE CL-4B GEL FILTRATION COLUMN - CHROMATOGRAPHY OF DOUBLE LABELLED $^3H/^{14}C$-HDL_3.** ^3H-CO, ^{14}C-TG double labelled HDL_3 (8ml, 0.17mg/ml; 1500dpm/μl ^3H; 140 dpm/μl ^{14}C) was loaded onto a 100ml Sepharose CL-4B column and eluted at 10ml/hr with Tris-saline buffer. Fractions (2.5ml) were sampled (50μl) and counted in a liquid scintillation counter.

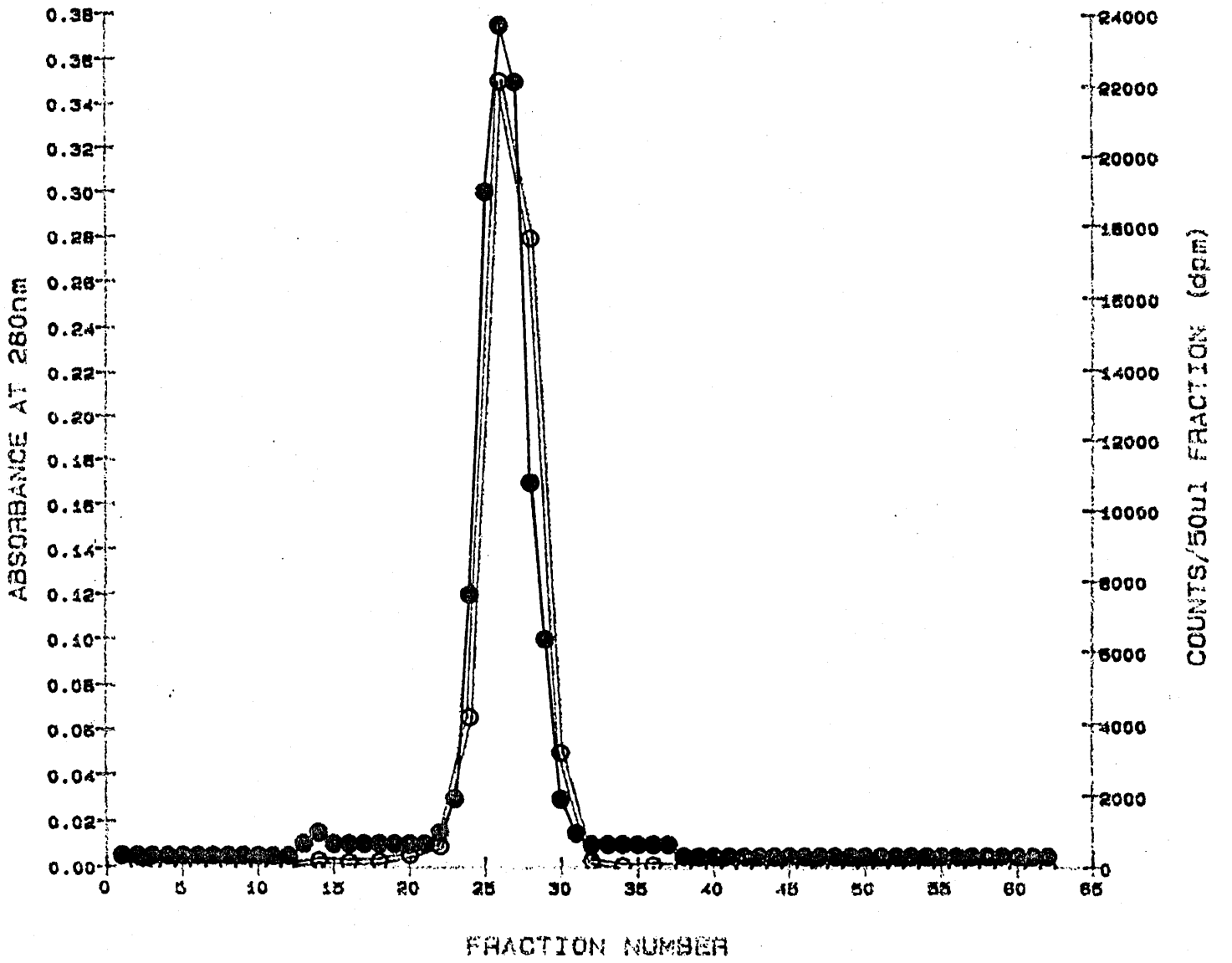


Fig 3.4

PROTEIN
RADIOACTIVITY

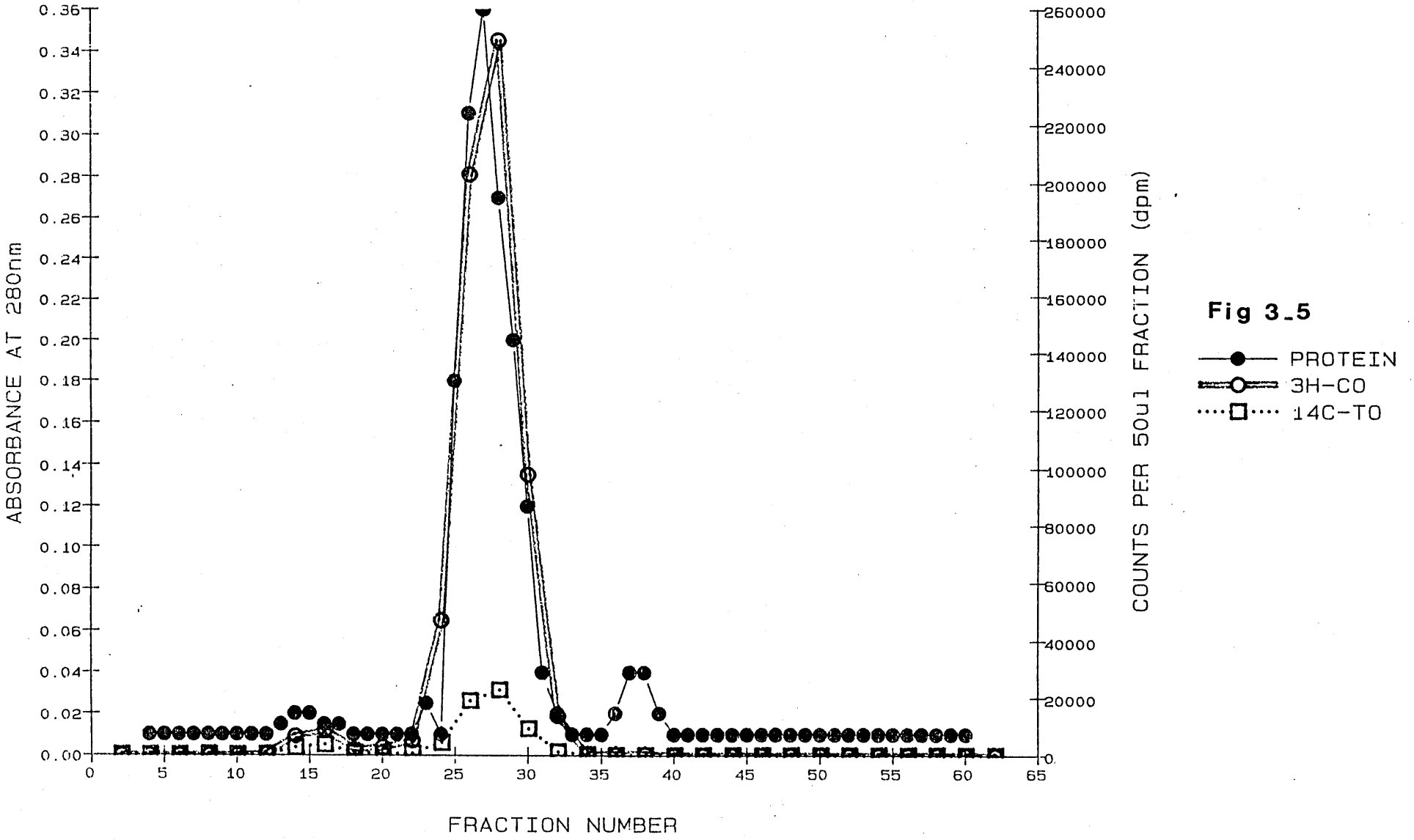

ABSORBANCE AT 280nm
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25
30
35
40
45
50
55
60
65
FRACTION NUMBER
COUNTS PER 50ul FRACTION (dpm)
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
PROTEIN
3H-CO
14C-TO

Fig 3_5

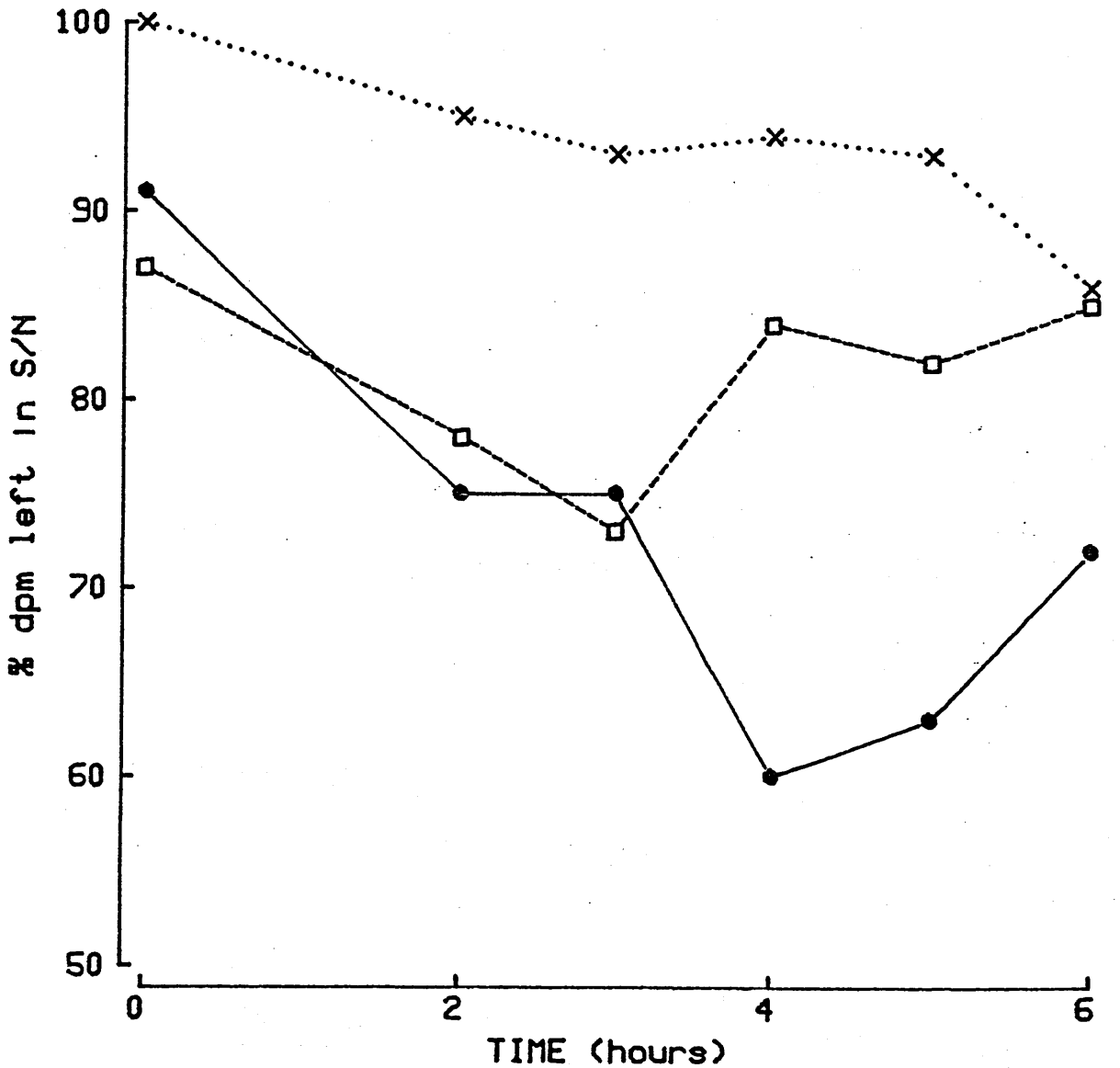


FIGURE 3.6 CETP BLANK TIME COURSE - REMOVAL OF CONTAMINATING ACTIVITY FROM HDL_3. ^3H-HDL_3 (5μg) and LDL/VLDL (8μg) were incubated together for up to 6 hours at 42°C and then separated by heparin manganese precipitation. (●) - HDL_3 was harvested at d=1.215g/ml and then passed through a Sepharose CL-4B gel filtration column, (□) HDL_3 was harvested at d=1.215g/ml and then subjected to centrifugation at d=1.125g/ml followed by centrifugation at d=1.215g/ml, (×) HDL_3 was subjected to centrifugation at d=1.125g/ml and d=1.215g/ml and was then passed down a gel filtration column.

itself to rapid processing of large numbers of samples so it was on this technique that effort was concentrated. The ultracentrifugation method however is useful to check the validity of the precipitation technique since 1) the methods of separation are based on different physical principles and 2) ultracentrifugation is an accepted method for separating high and low density lipoproteins.

The precipitation method used was that described by Tollefson and Albers [Warnick, G.R. & Albers, J.J. (1978), Tollefson, J.H. & Albers, J.J. (1986)]. Heparin and manganese form a complex with lipoproteins. Complex formation is favoured with larger lipoproteins or larger sulphated polysaccharides. The manganese cation is one which forms particularly stable complexes. Selective lipoprotein precipitation can be achieved by altering the sulphated polysaccharide to cation ratio. HDL_3 which contains little or no apoE is used in these assays since this protein may bind to heparin. Plasma is also added to improve the separation by adding bulk cold lipoproteins. An amount of bulk plasma (150µl) greater than that recommended by the published method was used as it led to better separation (Tollefson, personal communication). In practice some HDL_3 is precipitated and some LDL/VLDL is not. Experiments were carried out using labelled HDL_3 or LDL/VLDL to test which were the optimum precipitation conditions in our hands. Table 3.3 shows precipitations carried out in different containers with different final assay volumes. The best precipitation, with 85% ^{3}H-HDL_3 counts left in supernatant, occurred when the precipitation was carried out in microtitre plates. These plates were also convenient for carrying out large numbers of assays.

Using ^{3}H-HDL_3 to calculate the proportion of counts left behind in the supernatant, various concentrations of heparin manganese reagent were tested for their effectiveness. From the results shown in Table 3.4, final concentrations of 213U/ml heparin: 0.085M $MnCl_2$ were decided upon as the conditions for optimum separation. Under these conditions 11% HDL_3 was precipitated (though this was later improved, see Table 3.5) and 1% LDL/VLDL was left in solution.

Once a concentration of precipitating reagent was decided upon, the other assay components were added and precipitation carried out at time zero to see whether the other assay components would interfere with the precipitation. Using ^{3}H-HDL_3 only, counts not precipitated could be refined to >95% and using ^{3}H-LDL/VLDL only counts not precipitated could be refined to <1% (Table 3.5). This improvement in definition probably reflects improved sampling. In the course of determining variability between operators (data not shown) it was found that the biggest source of error was in sampling the supernatant after precipitation probably because the pellet is soft. Using one labelled substrate and adding the other substrate unlabelled, these precipitation blanks were not altered greatly by the presence of the other substrate (Table 3.5).

CONTAINER	ASSAY VOLUME (μl)	BLANK (dpm in S/N)
GLASS TUBE	600	66%
MICROFUGE TUBE	600	69%
MICROTITRE PLATE	120	85%

TABLE 3.3 **CHOICE OF CONTAINERS FOR CETP ASSAY.** ^{3}H-HDL_3 (0.04μg/μl final concentration) and LDL/VLDL (0.07μg/μl final concentration) were made to the appropriate final volume with Tris-saline buffer in either glass tubes, microfuge tubes or microtitre plates. Heparin manganese reagent (266U/ml heparin: 0.106M $MnCl_2$) was used to precipitate the lower density lipoproteins. Percentage counts remaining in the supernatant were calculated.

HEPARIN MANGANESE REAGENT (final concentration heparin:$MnCl_2$ U/ml:M)	HDL BLANK (% dpm in pellet using ^3H-HDL_3)	LDL/VLDL BLANK (% dpm in S/N using ^3H-LDL/VLDL)
106:0.043	9 + LPDS 7	3 + LPDS 23
160:0.064	12 + LPDS 9	2 + LPDS 2
213:0.085	11 + LPDS 12	1 + LPDS 1
239:0.096	12 + LPDS 12	0.9 + LPDS 1
266:0.106	22 + LPDS 13	0.7 + LPDS 0.7

TABLE 3.4 **REFINEMENT OF THE HEPARIN MANGANESE PRECIPITATION.** HDL_3 (5μg) and LDL/VLDL (8μg) in the presence or absence of LPDS (10μl) were made up to a volume of 60μl with Tris-saline buffer. Carrier plasma (150μl) was added and then 25μl of a dilution of a 2500U/ml: 1M heparin:$MnCl_2$ solution. Microtitre plates were left to stand for 25 minutes on ice and then centrifuged at 2800rpm for 25 minutes. The supernatant was sampled (150μl) and counted. For determination of the HDL blank, ^3H-HDL_3 was incubated with unlabelled LDL/VLDL; and for the LDL/VLDL blank, unlabelled HDL_3 was incubated with ^3H-LDL/VLDL.

REAGENTS	% dpm in S/N
^{3}H-HDL$_3$ only	102
^{3}H-LDL/VLDL only	0.7
^{3}H-HDL$_3$ and cold LDL/VLDL	96
cold HDL$_{TOTAL}$ and ^{3}H-LDL/VLDL	0.9

TABLE 3.5 **HEPARIN MANGANESE PRECIPITATION.** HDL$_3$ (5μg) and LDL/VLDL (8μg) were made to a final volume of 60μl with Tris-saline buffer. To precipitate, plasma (150μl) and heparin manganese reagent (25μl of 2000 :U/ml heparin:0.8M $MnCl_2$) were added. Supernatants were sampled after precipitation and radioactivity determined by scintillation counting.

COMPONENT	HDL BLANK (dpm in pellet)	LDL/VLDL BLANK (dpm in S/N)
BLANK	11%	0.8%
LPDS (dialysed against PBS)	22%	1.3%
LPDS (dialysed against Tris-saline)	13%	0.7%
0.7mM DTNB	6%	0.9%

TABLE 3.6 **EFFECT OF ASSAY COMPONENTS ON PRECIPITATION.** Precipitations were carried out as for Table 3.5 but the incubations contained either buffer only, LPDS dialysed against phosphate buffered saline (10μl), LPDS dialysed against Tris-saline buffer (10μl) or 0.7mM DTNB.

As shown in Table 3.6 inclusion of DTNB (0.7mM) or LPDS which had been dialysed against Tris-saline buffer did not significantly affect precipitation at time zero. LPDS which had been dialysed against phosphate buffered saline doubled the amount of HDL_3 precipitated by the reagent. Manganese phosphate on its own can precipitate lipoproteins and this effect is probably interfering with the heparin mediated precipitation. Before CETP assay, all samples are dialysed against Tris-saline buffer.

3.3.2 Measurement of CETP Activity

Having prepared the substrates and defined the conditions for their separation CETP assays were carried out. Substrates were incubated together with a source of CETP in the presence of DTNB. DTNB, an LCAT inhibitor, was included so that transfer activity would not be influenced by the amount of CE synthesised by the enzyme. If there were greatly different amounts of LCAT between samples then it is possible that the specific activity of the donor HDL_3 may be affected by the amount of synthesis of cold CE. A concentration of 0.7mM was sufficient to inhibit LCAT (Fig 3.7). Concurrent with incubations containing a test CETP source, two control incubations were also run with every assay. One, a blank, had no CETP source and indicates the background rate of spontaneous (or contaminating) transfer taking place. This is always subtracted from the values obtained to give a true transfer rate. A second control incubation is that of a standard LPDS, included to try and maintain consistency between assays. Since the substrates used and the control CETP are all from different individuals it is important to try and rule out any variability between donors of the assay components affecting assay rates. To overcome this, new substrate preparations or control LPDSs were introduced one at a time, and before use, each new batch was tested in a control incubation and only used if they showed similar activity to the previous batch of reagents. Each assay is carried out in triplicate to avoid error introduced by bad sampling of supernatant as mentioned earlier. Coating the microtitre plates with BSA (as described in the methods) seemed to improve replication within an assay and so this was carried out as a general practice (data not shown).

Time courses were carried out to determine the period of time over which transfer was linear. Originally assays were carried out at 37°*C*. However it was found that it took approximately 10 hours to achieve 30% transfer, an assay duration that is not convenient. In order that an assay may be carried out easily within one day, activity was assayed at 42°*C* and took approximately 3 hours for an equivalent amount of protein to express 30% transfer. Fig. 3.8 illustrates the time course of the same amount of CETP at the different temperatures.

A typical standard protein curve and time course of activity for human LPDS are shown in Fig. 3.9. The protein curve is linear to 35% transfer and then transfer saturates. Originally amounts of substrates were varied relative to each other to optimise transfer rates. It was found that 5μg protein ^3H-HDL_3 and 8μg protein

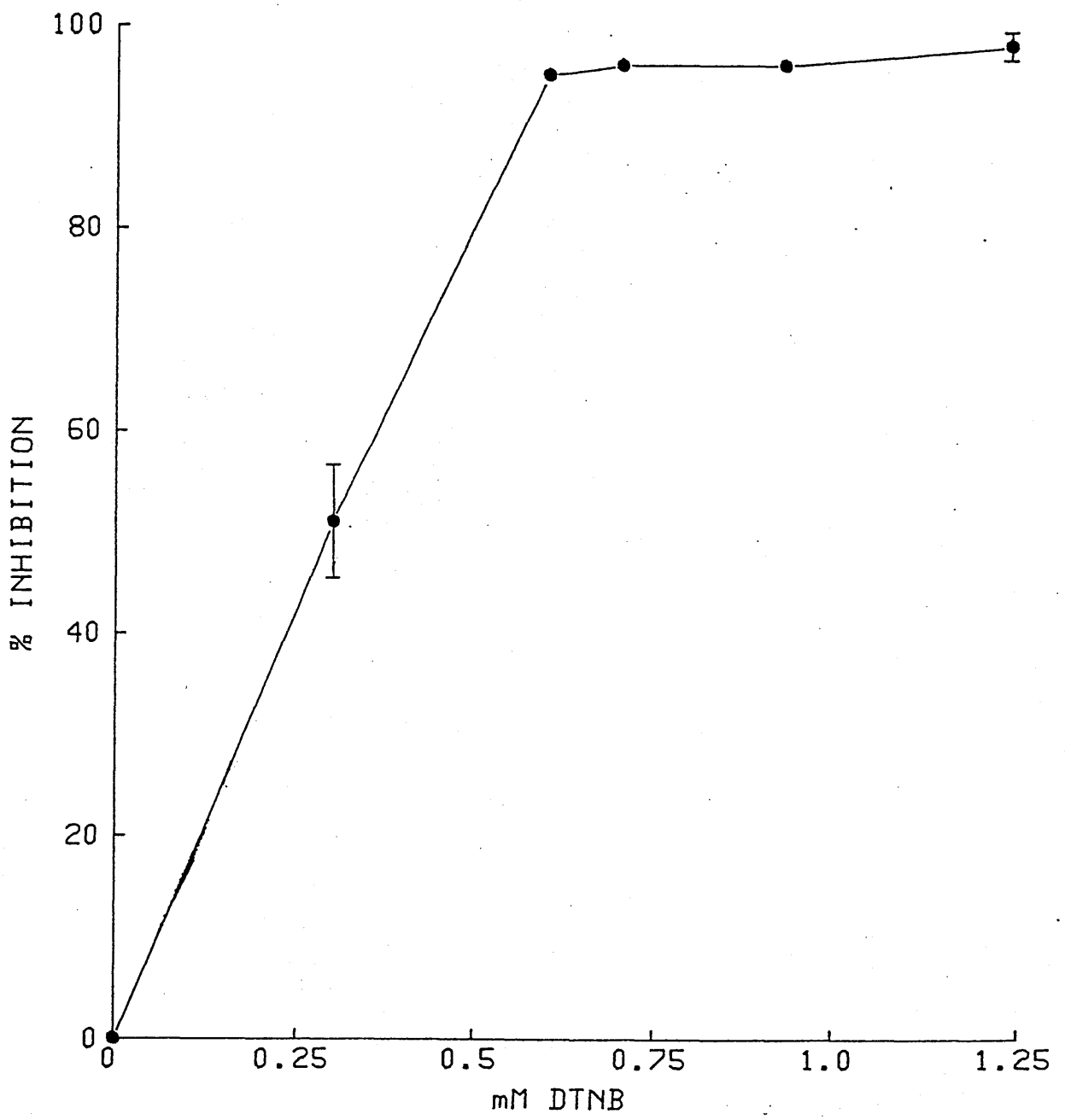


FIGURE 3.7 **LCAT ACTIVITY - DTNB CURVE.** LCAT assays were carried out as described in Methods but mercaptoethanol was omitted from the assay buffer. DTNB in water was added to the assays at various concentrations.

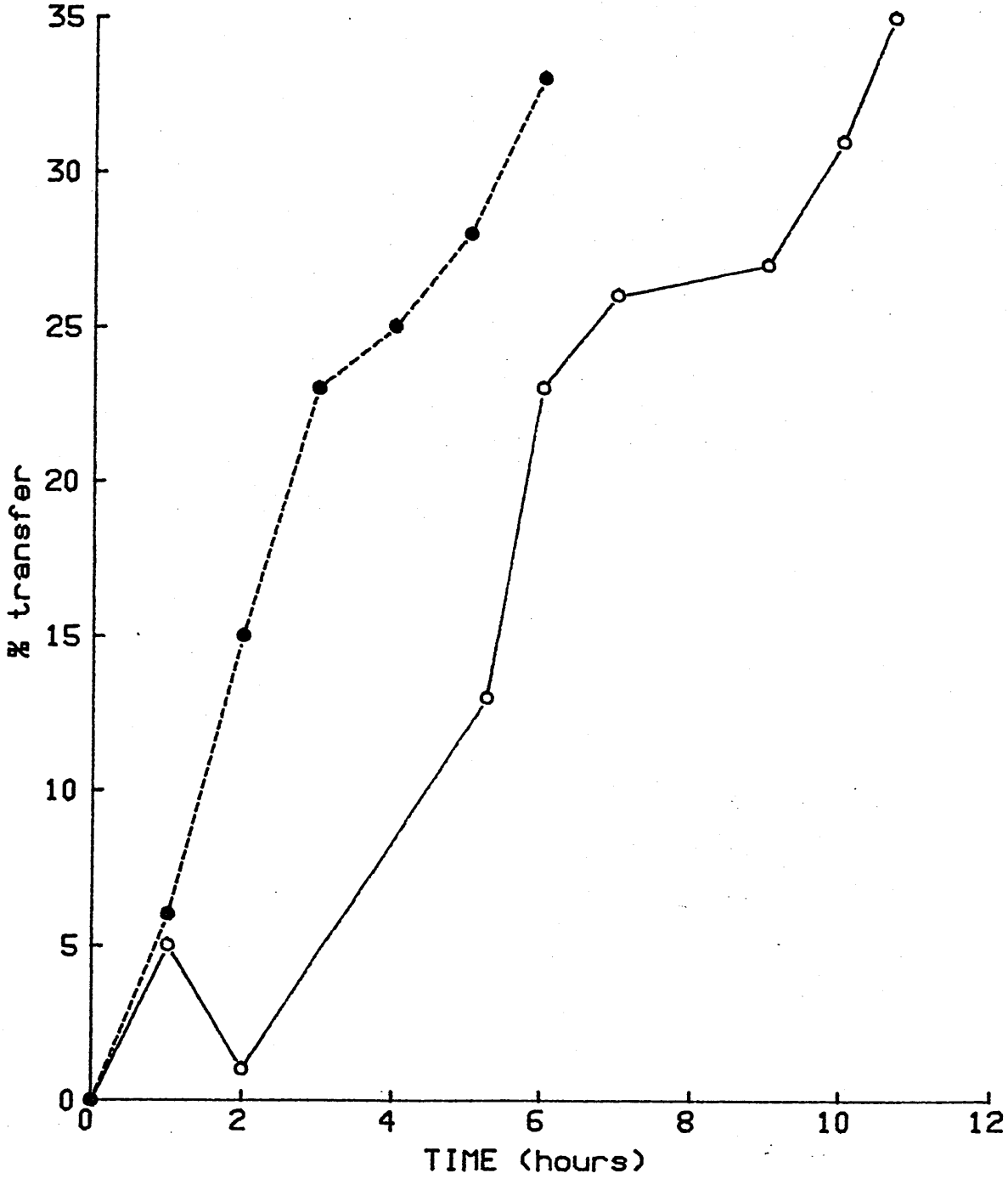


FIGURE 3.8 CETP TIME COURSE AT 37°C AND 42°C. CETP assays were carried out in the presence of LPDS at 37°C (○) or 42°C (●).

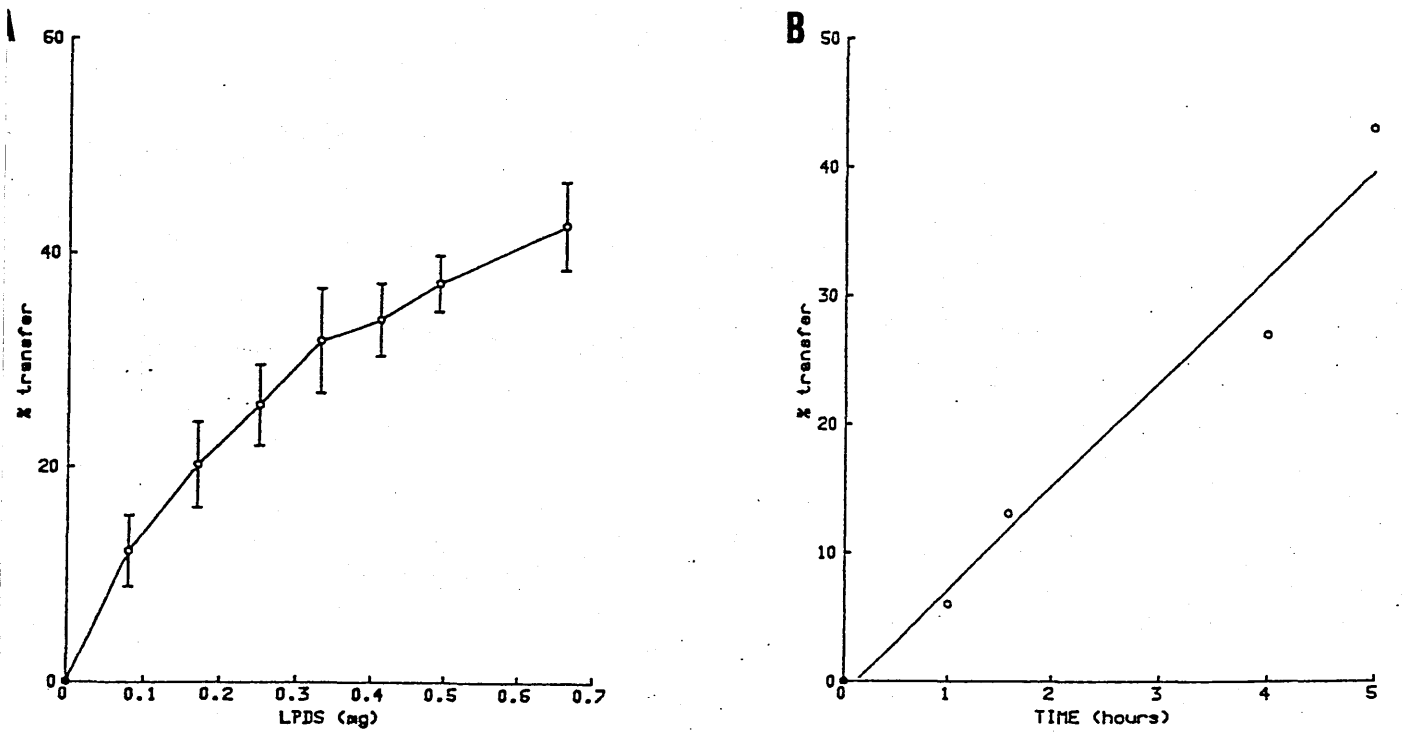


FIGURE 3.9 **CETP PROTEIN CURVE AND TIME COURSE - 18/8/89 LPDS.** A) CETP assays were carried out in the presence of different amounts of human LPDS (18/8/89) for 2.5 hours at 42°*C*. This graph represents the average of five separate protein curve determinations. B) Assays were carried out in the presence of 0.16mg human LPDS (18/8/89) for various lengths of time at 42°*C*.

LDL/VLDL were suitable concentrations of substrates to give maximal transfer for a given CETP sample. Fig. 3.9A is the average of 5 experiments carried out on separate days. As can be seen from the standard deviation bars variation can be as great as +\- 5% transfer. To determine CETP activities in unknown samples, activity was measured at at least three different protein concentrations at a dilution giving activity well within the range of the standard curve. Activity was calculated as % transfer per microlitre using regression analysis to calculate the slope. To account for any dilution which may have occurred during sample preparation, activity was then expressed as % transfer per microgram of sample protein. The time course. Fig. 3.9B, shows linearity to 2.5 hours at 42°C and this was chosen as the assay incubation time.

Assays carried out using HDL_3 substrate labelled either with ^3H-cholesteryl oleate or ^{14}C-triglyceride, or both show very similar transfer rates, Fig. 3.10. Thus it appears that triglyceride is being transferred in similar fashion to CE and transfer of both can be seen simultaneously. The activity measured here is an exchange activity, and it can be seen that both CE and TG can be transferred from HDL to lower density lipoproteins. However there is no indication as to how much mass of CE or TG will end up in the lower density lipoproteins since back transport from LDL/VLDL to HDL_3 is not measured.

Fig. 3.11 shows the effect of keeping the concentration of one substrate constant and varying the concentration of the other. When LDL/VLDL concentration is kept constant and HDL_3 is increased then the amount of CE transferred increases in a linear fashion up to at least 8μg (Fig. 3.11A). This shows that the amount of HDL_3 used in the assay is rate limiting. When the amount of HDL_3 is held constant and LDL/VLDL is increased then the activity starts to saturate at about 6μg LDL/VLDL, (Fig. 3.11B). Thus this substrate is not rate limiting. If this data is expressed as a double reciprocal plot (Fig. 3.11C), then the values for the Km and Vmax obtained are 5.2μg and 44.4% transfer respectively.

A comparison of the rates of transfer to different low density lipoprotein fractions was carried out (Fig. 3.12). LDL was the best acceptor followed by an LDL/VLDL mix and then by VLDL. It might be expected that VLDL would be a better donor than LDL since it has a greater CE plus TG to protein ratio. It is possible that this particular VLDL may not have been a good acceptor because it had low triglyceride if it was prepared from blood collected from a fasting subject, or it had an unusual composition or its protein content was underestimated due to an interfering effect of TG in a Bradford assay. Smaller particles have a larger surface to volume ratio and if interaction of CETP with the lipoprotein surface is important then LDL may be a better acceptor because it is smaller than VLDL. Despite LDL being a better acceptor, the use of LDL/VLDL was continued as in the original method [Tollefson, J.H. & Albers, J.J. (1986)] since it was thought that the LDL/VLDL might represent a more physiological substrate since CETP can transfer to both these lipoproteins *in vivo*.

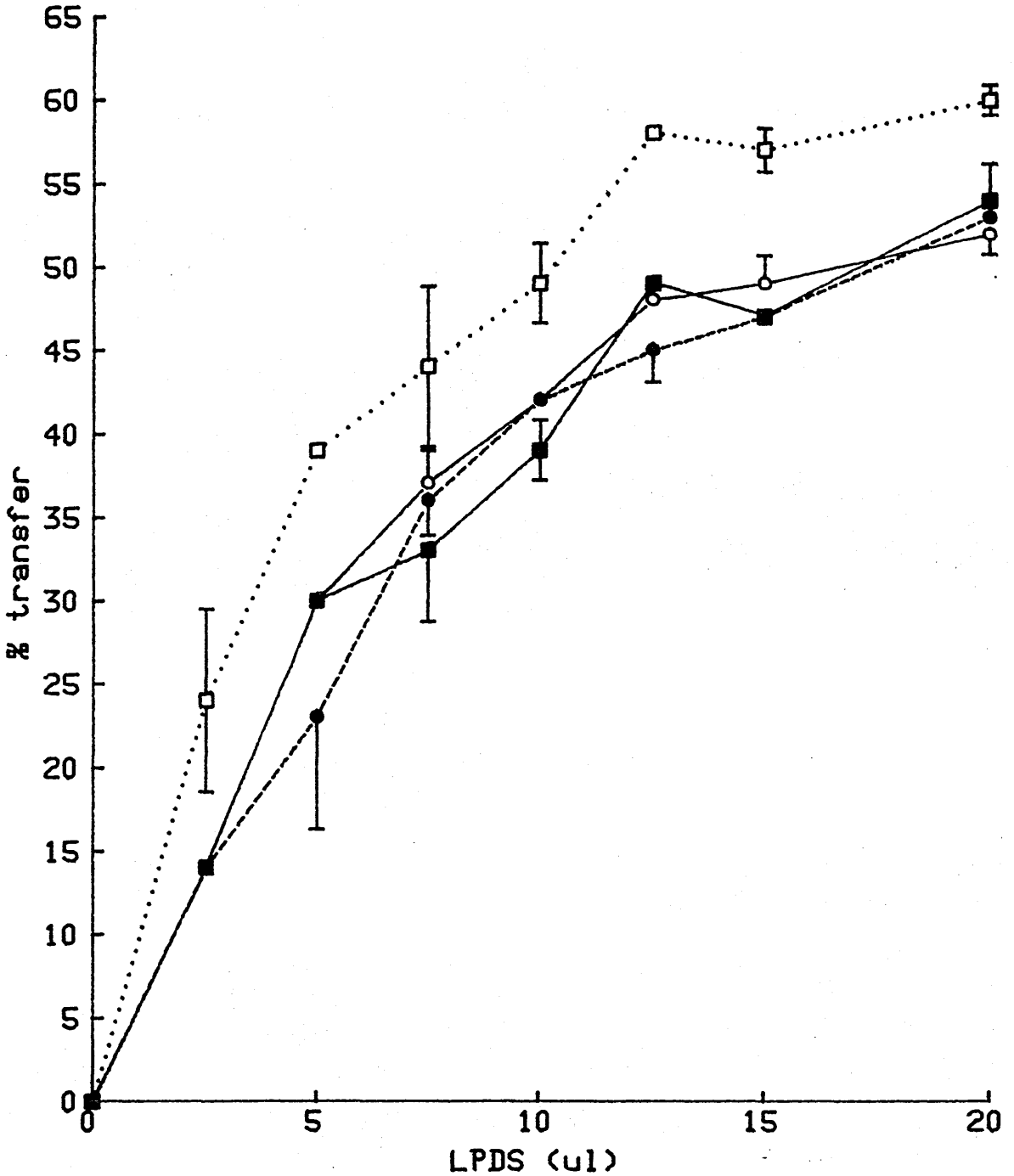
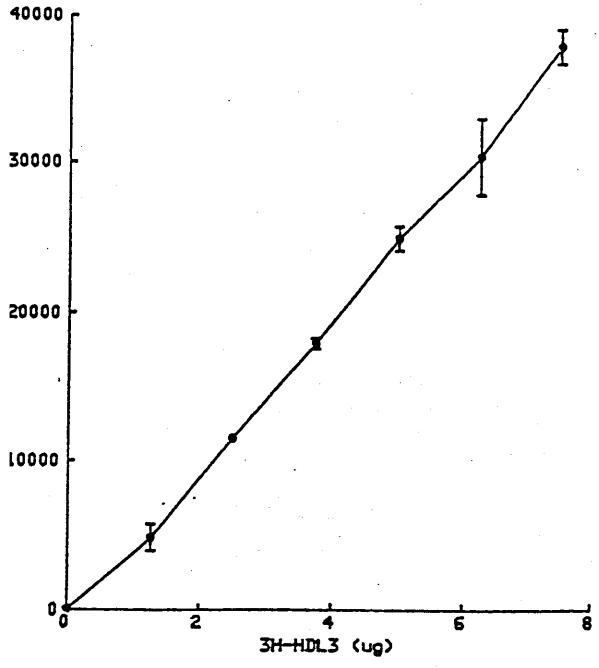
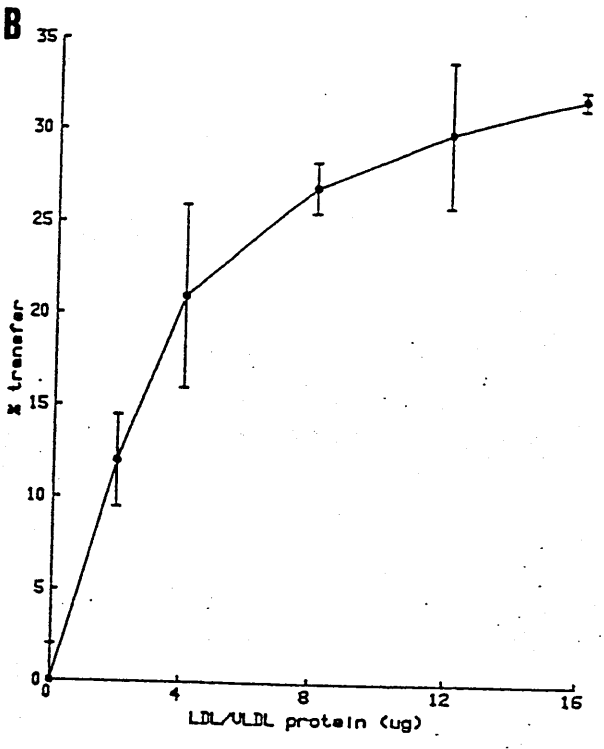


FIGURE 3.10 CETP PROTEIN CURVES WITH ^{3}H-CO, ^{14}C-TO AND ^{3}H-CO/^{14}C-TO HDL_3. Similar CETP assays were carried out for 2.5 hours at 42°C with HDL_3 (5μg) and LDL/VLDL (8μg). (○) ^{3}H-CO HDL_3, (●) ^{14}C-TO HDL_3, (□) ^{3}H-CO/^{14}C-TO HDL_3 - ^{3}H counts measured and (■) ^{3}H-CO/^{14}C-TO HDL_3 - ^{14}C counts measured.





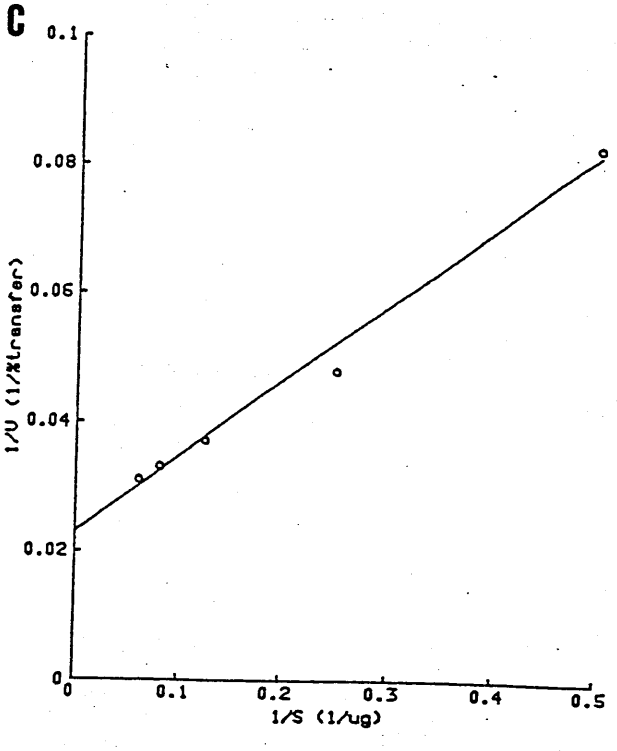


FIGURE 3.11 **CETP ASSAY, VARIATION OF SUBSTRATE.** A) A CETP assay was carried out with variable amounts of ^{3}H-HDL$_3$ (0-7.5μg) and constant LDL/VLDL (8μg). Assays were in triplicate and activity was calculated as the amount of ^{3}H counts transferred from the supernatant containing HDL$_3$ i.e. the amount of counts added to an assay minus the amount remaining in the supernatant. B) A CETP assay was carried out with variable amounts of LDL/VLDL (0-16μg) and with a constant amount of ^{3}H-HDL$_3$ (5μg). Percent transfer was calculated in the usual way. Assays were in triplicate. C) A double reciprocal plot is shown of the data found in Fig. 3.11B. V_{MAX} = 44.4 % transfer, Km = 5.2μg.

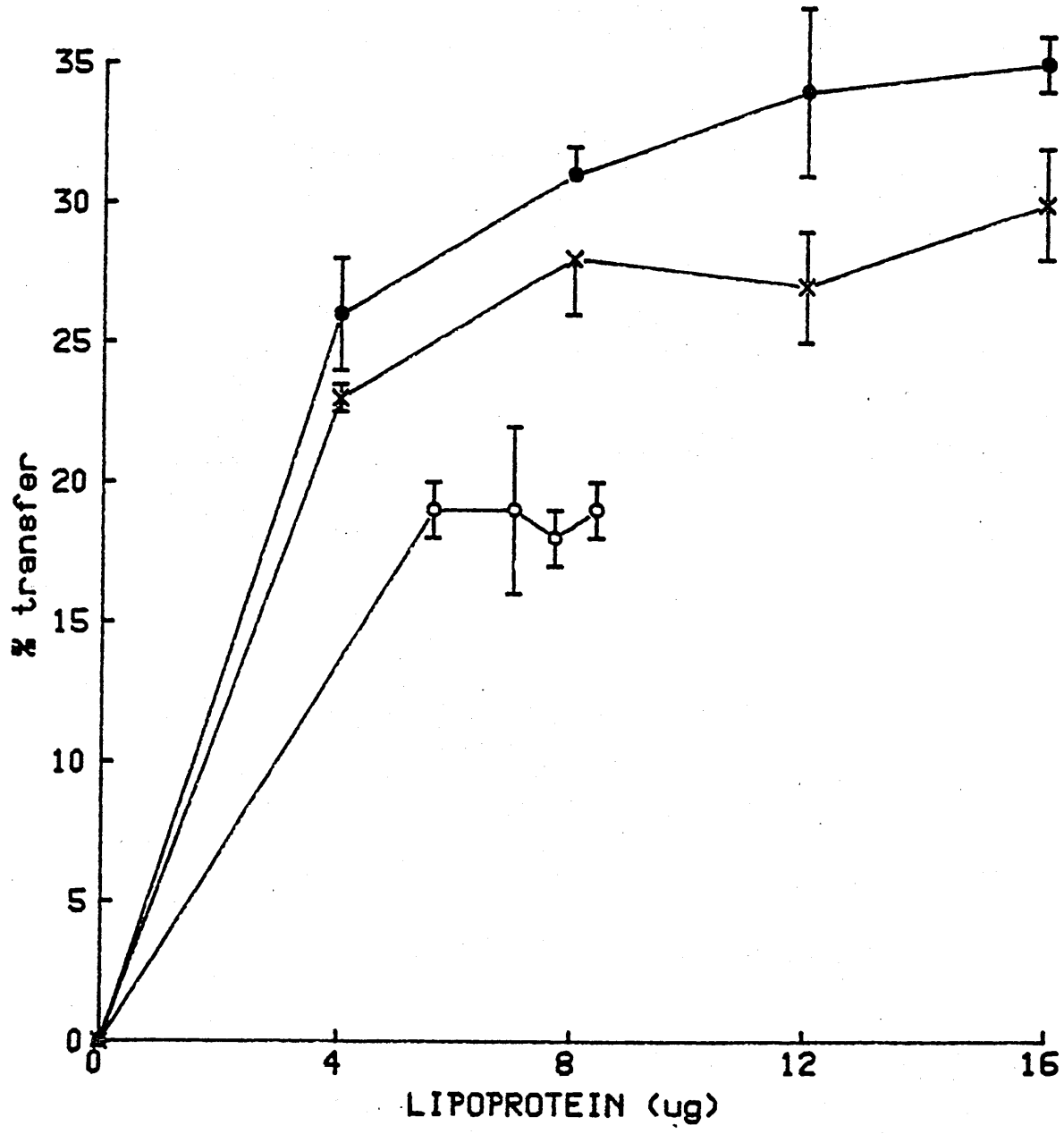


FIGURE 3.12 CETP ASSAY - DIFFERENT ACCEPTORS. CETP assays contained ^{3}H-HDL_3 (5μg) and variable amounts of VLDL (○) 0-8.4μg, LDL (●) 0-16μg and LDL/VLDL (×) 0-16μg. Assays were in triplicate.

There have been reports of an assay which uses a lipoprotein bound to a solid support as a donor [Sparks, D.L. *et al* (1987)]. This type of substrate has the advantage that any pairs of donor and acceptor lipoproteins can be used, including using the same lipoproteins as donor and acceptor, and also the donor and acceptor pairs are easily separated by a short centrifugation. An attempt was made to develop such a solid support assay using radiolabelled HDL bound to Sepharose CL-4B. However although linear transfer rates were observed, about ten times more CETP protein was required to achieve similar rates of transfer to those found in the usual CETP assay i.e. 5mg protein to reach 30% transfer in 2.5 hours compared to 0.35mg in the usual assay. Any further attempts to develop this technique were therefore abandoned.

3.3.3 Validation of the Precipitation Technique Using Ultra-centrifugation

In order to confirm the validity of using precipitation to separate donor and acceptor particles, experiments were carried out directly comparing transfer rates measured by the precipitation technique and transfer rates measured by the ultra-centrifugation technique i.e. loss of radioactivity from the high density lipoprotein fraction and the appearance of radioactivity in the lower density lipoprotein fraction. A protein curve (Fig. 3.13A) showed similar transfer rates whether transfer was measured by precipitation or by ultracentrifugation separation. This was also the case for a time course of transfer activity (Fig. 3.13B). Thus using an entirely different technique for separating donor and acceptor particles similar transfer rates can be measured. Since the precipitation technique is much less time consuming and is adaptable to coping with large sample numbers, this technique was routinely used.

3.3.4 A More Sensitive CETP Assay

In some instances, especially in the case of measuring CETP activity in cell media, the sensitivity of the above described assay was not great enough to detect the small amounts of activity present. In order to improve the sensitivity the assays were incubated for a much longer period of time. Since activity is linear to 30% transfer it ought to be possible to incubate the low activity samples long enough to reach this level of activity and the increase in activity should be linear. This was found to be the case. As shown in Fig. 3.14 a 1/4 dilution of a standard LPDS reached 30% transfer in 2 to 3 hours whereas a 1/40 dilution of the same LPDS took approximately 10 hours to reach 30% transfer, but the progression was linear. Thus this long duration CETP assay could easily detect 10 times less activity than the usual CETP assay. The problem with this long incubation assay was that the blank rate increased. Obviously if there is still any CETP associated with the substrates then this activity will be amplified in the more sensitive assay also. It was found that inclusion of a final concentration of 3% BSA in the assay improved replication between triplicates. It is possible that during prolonged incubation at 42°C the lipoproteins begin to degenerate which may affect their precipitation by heparin

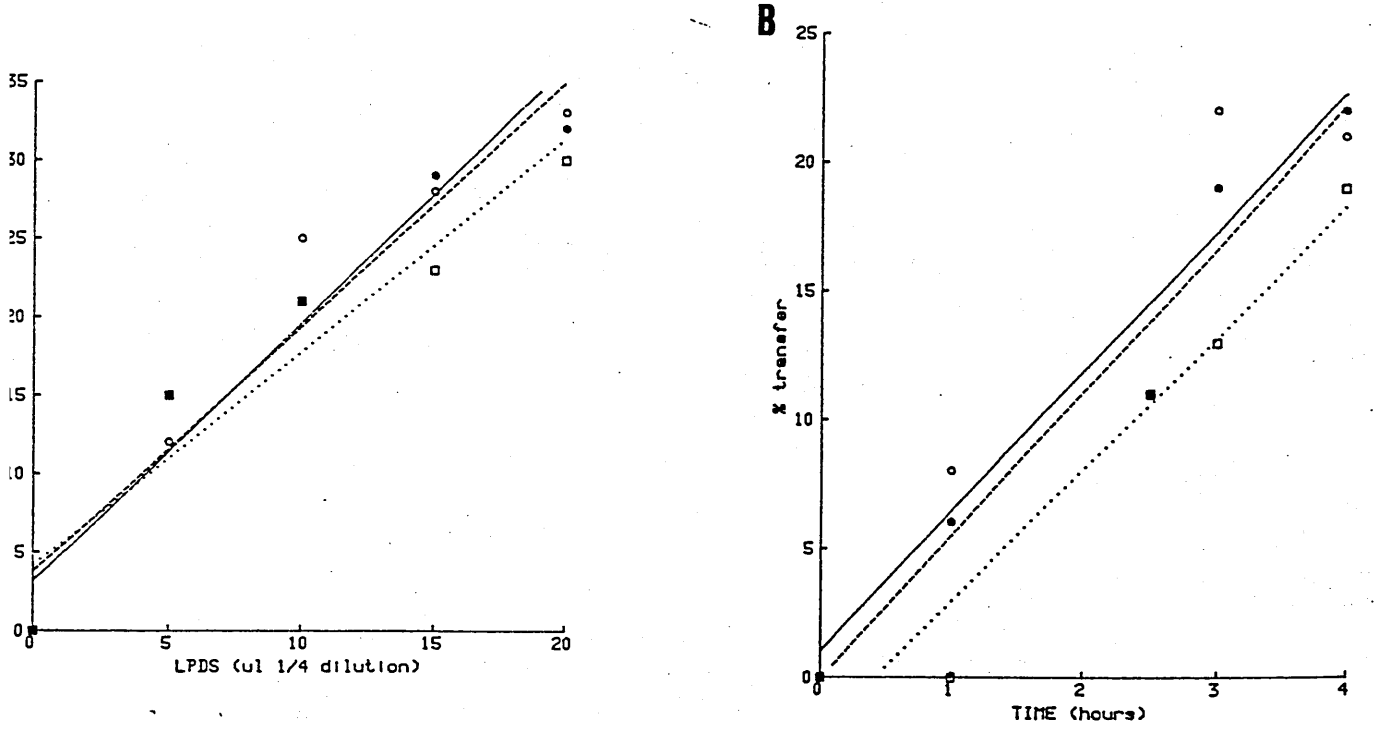


FIGURE 3.13 CETP ASSAY USING CENTRIFUGATION TO SEPARATE LIPOPROTEINS. Two identical protein curves (A) or two identical time courses (B) were carried out in parallel. At the end of the incubation one of the pairs of assays was stopped in the usual manner and the lipoproteins separated by precipitation (○). In the second assay a 50µl sample was placed in a one ml polycarbonate centrifuge tube. A d=1.182g/ml NaBr solution (25µl) was added to bring the density to 1.063g/ml and then the mix was overlaid with a d=1.067g/ml NaBr solution. Tubes were centrifuged for 3 hours at 100000rpm in a Beckman TL100 Tabletop centrifuge. At the end of the centrifugation the top and bottom 300µl were sampled and counted. The radioactivity in the top fraction (●) represented the lower density lipoproteins (LDL/VLDL) and the bottom fraction (□) represented the high density lipoprotein fraction.

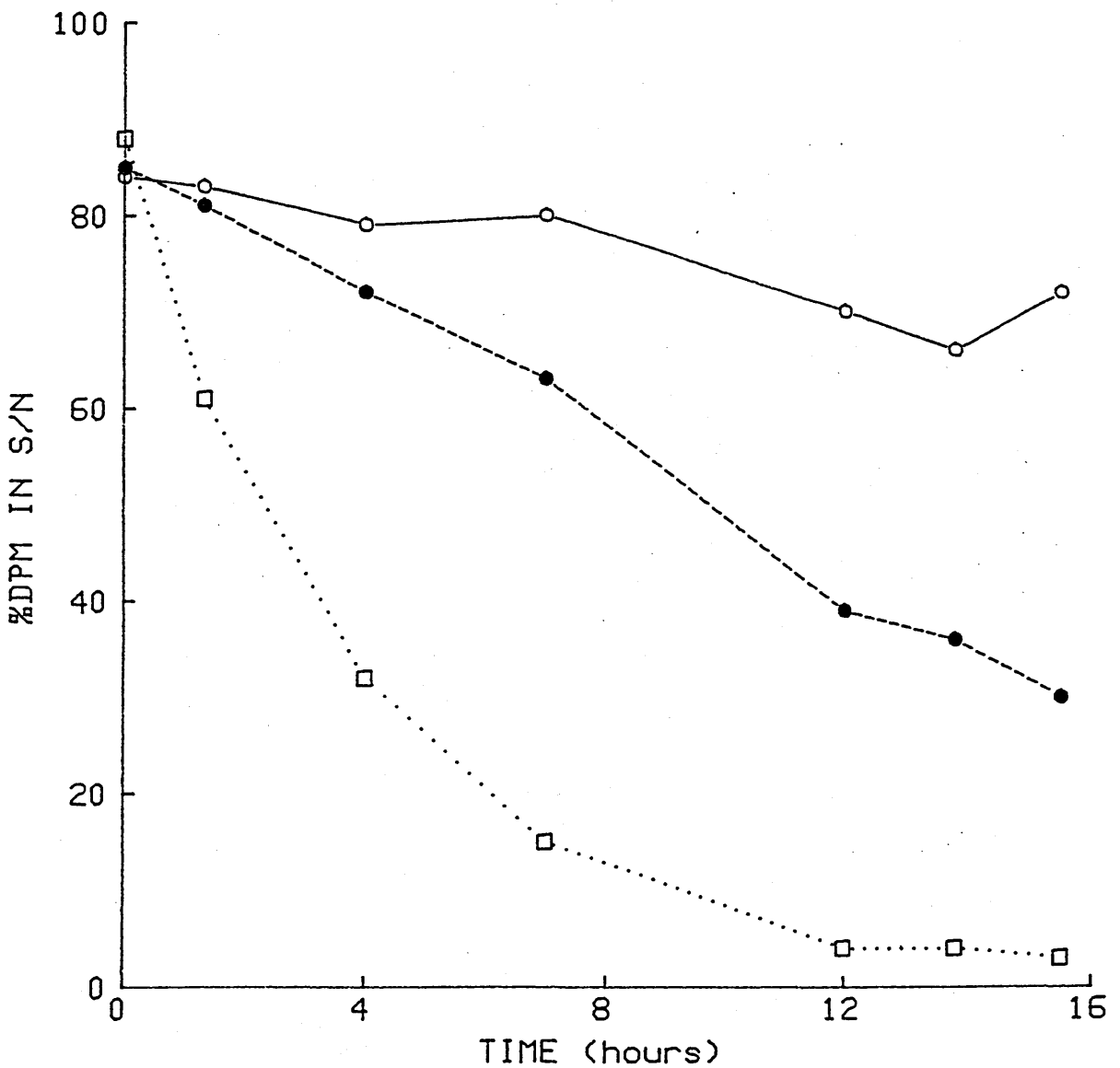


FIGURE 3.14 . SENSITIVE CETP ASSAY - LOSS OF DPM FROM THE SUPERNATANT. A two times volume CETP assay was carried out in an microfuge tube containing (○) blank, (●) 1/40 dilution LPDS (25/7) or (□) 1/4 dilution LPDS (25/7). The assays were incubated for up to 16 hours at 42°C. The assay was stopped with 300µl plasma and 50µl heparin manganese reagent.

manganese. Inclusion of BSA may improve the stability of the lipoproteins. An 8 hour assay incubation time was chosen as a convenient length for this sensitive assay. A protein curve is shown in Fig. 3.15. As can be seen from the standard deviations this assay is much more variable than the usual CETP assay and therefore much less accurate. Nevertheless it can prove useful when trying to discern whether a small amount of activity is present or not.

3.3.5 Properties of the Measured CETP Activity

In order to ascertain whether the activity measured by the CETP assay was the same as that described in the literature, the properties of the measured CETP activity were examined. Properties that have been reported include species variation [Ha, Y.C. & Barter, P.J. (1982)], apoAI effects [Son, Y-S. C. & Zilversmit, D.B. (1984)] and heat stability [Tollefson, J.H. & Albers, J.J. (1986)].

Different species have been reported to demonstrate widely varying plasma levels of CETP activity ranging from very low in the rat to very high in rabbits. CETP activity was measured in LPDP samples from seven mammalian species; rabbit, sheep, human, rat, pig, marmoset and mouse. As predicted the activities measured fell into three groups (Fig. 3.16). Pigs, rats and mice had low plasma CETP activity; human and marmoset LPDP had intermediate activity and rabbit LPDP had high activity. This activity profile reflects that reported in the literature.

ApoAI has been reported to inhibit CETP activity. ApoAI inhibition could be measured to extents similar to those published in the literature. It was noticed however that apoAI from different donors inhibited to greatly different extents (Fig. 3.17). After this work was completed reports appeared in the literature of an inhibitor of CETP [Tollefson, J.H. *et al* (1988)]. This inhibitor was purified from HDL and had molecular weight 29000. It is possible that the inhibition of CETP by apoAI may be due to contamination of the apoAI preparations with some of this inhibitor. ApoAI is very close in molecular weight (28000) to the inhibitor protein and since apoAI is often purified by gel filtration it seems reasonable that apoAI preparations may be contaminated with this inhibitor protein. The apoAI used here was purified by gel filtration.

CETP is stable to heat treatment of one hour at 56°*C* whereas LCAT, another plasma enzyme is not. LPDP samples were incubated at 56°*C* for one hour and then CETP and LCAT activities were measured in treated and control samples. CETP activity was unaffected by heat treatment (Fig. 3.18A) whereas LCAT activity was totally inhibited by heat treatment (Fig. 3.18B).

Since CETP is relatively heat stable we sought to make use of this fact to try and separate CETP and CETP inhibitor activities. The measurement of plasma CETP activity is complicated by the presence of the CETP inhibitor protein. Any activity measured is the net result of CETP protein plus inhibitor protein and therefore is not a direct measure of how much CETP protein is there. If the two activities can

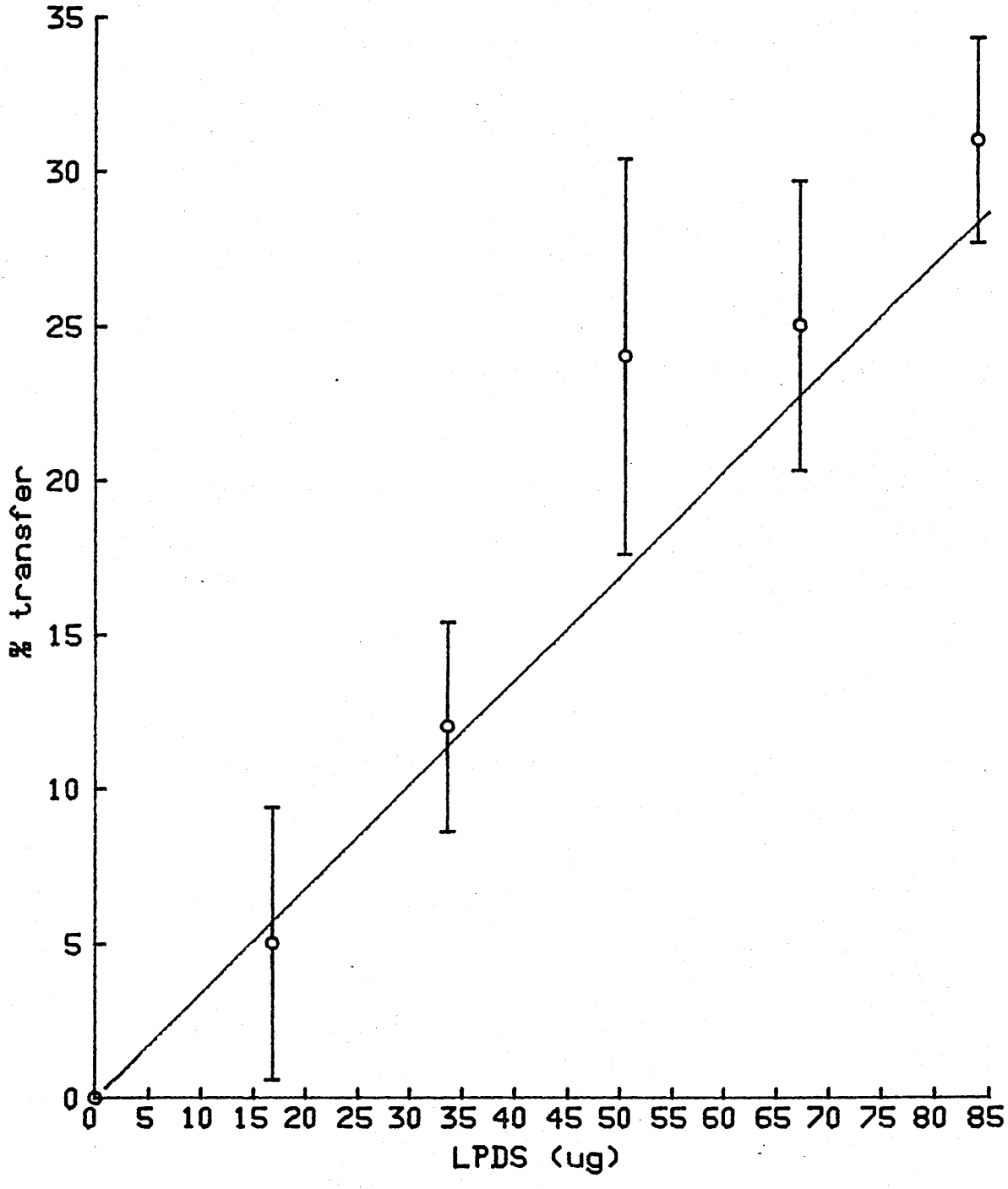


FIGURE 3.15 **SENSITIVE CETP ASSAY - PROTEIN CURVE, 8 HOURS.** A CETP protein curve was carried out for 8 hours using the conditions described in Fig. 3.14.

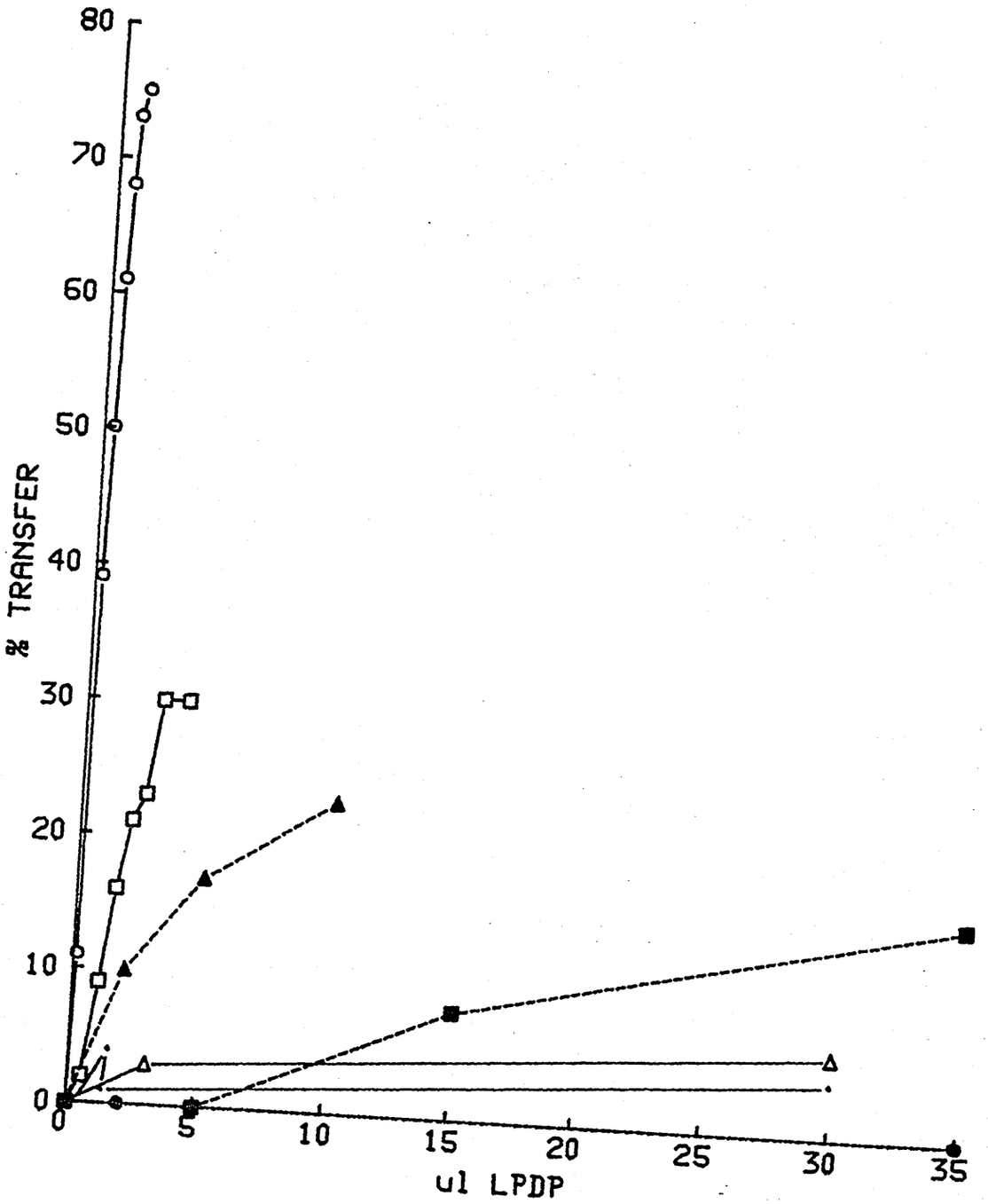


FIGURE 3.16 **CETP ACTIVITIES IN LPDP FROM SEVERAL ANIMAL SPECIES.** LPDP was prepared from the serum of the rabbit (○), sheep (●), human (□), rat (■), pig (△), marmoset (▲) and mouse (•) as the d>1.215g/ml fraction of plasma. CETP assays were carried out on these LPDPs for 2.5 hours at 42°C.

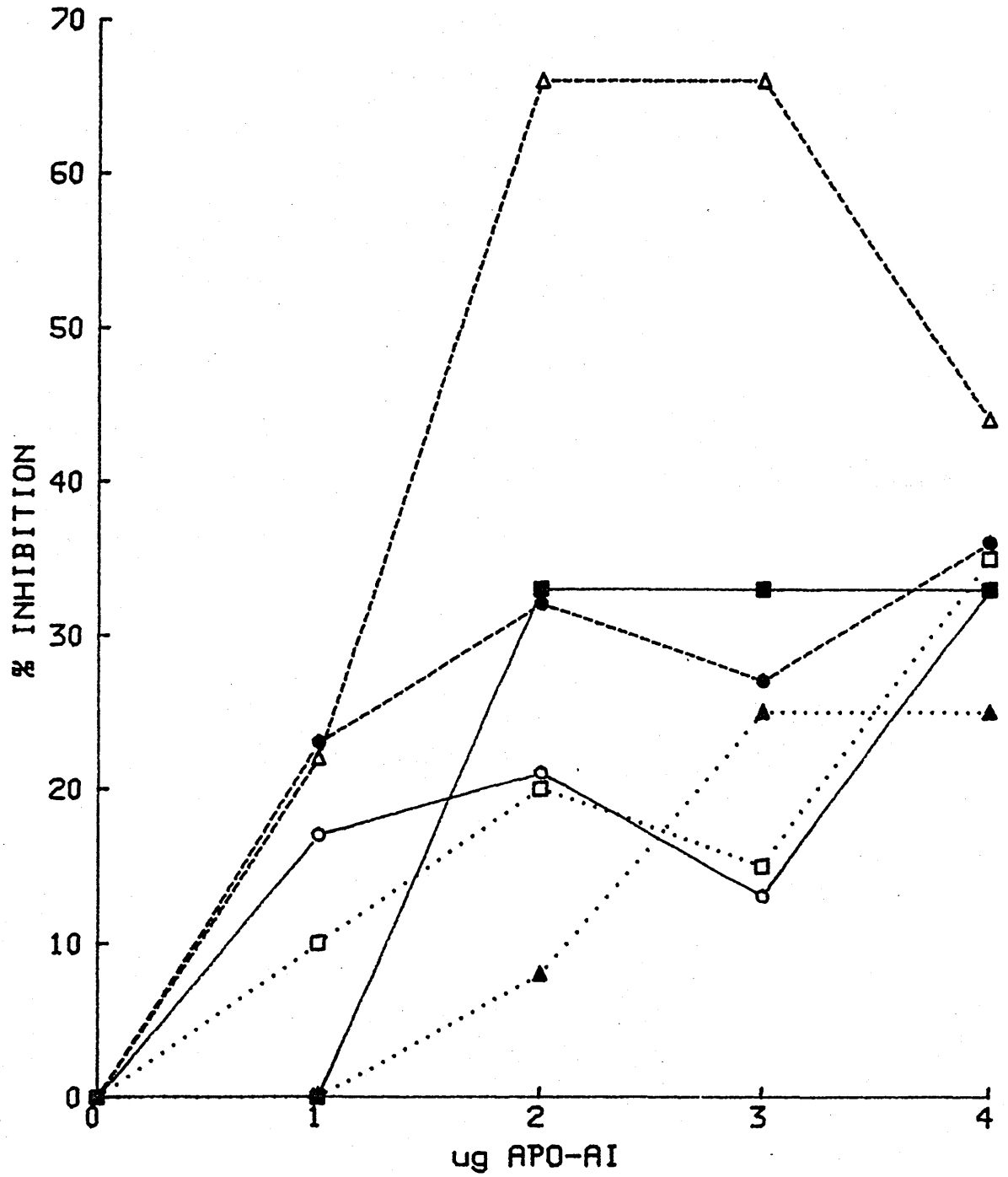


FIGURE 3.17 **APO-AI INHIBITION OF CETP - DIFFERENT APO-AI DONORS.** Apo AI from 6 human donors was received as a gift from Philip Stewart, Glasgow Royal Infirmary. Apo AI (0-4μg) was tested for its inhibitory effect in a CETP assay. (○) donor 1, (●) donor 2, (□) donor 3, (■) donor 4, (△) donor 5 and (▲) donor 6.

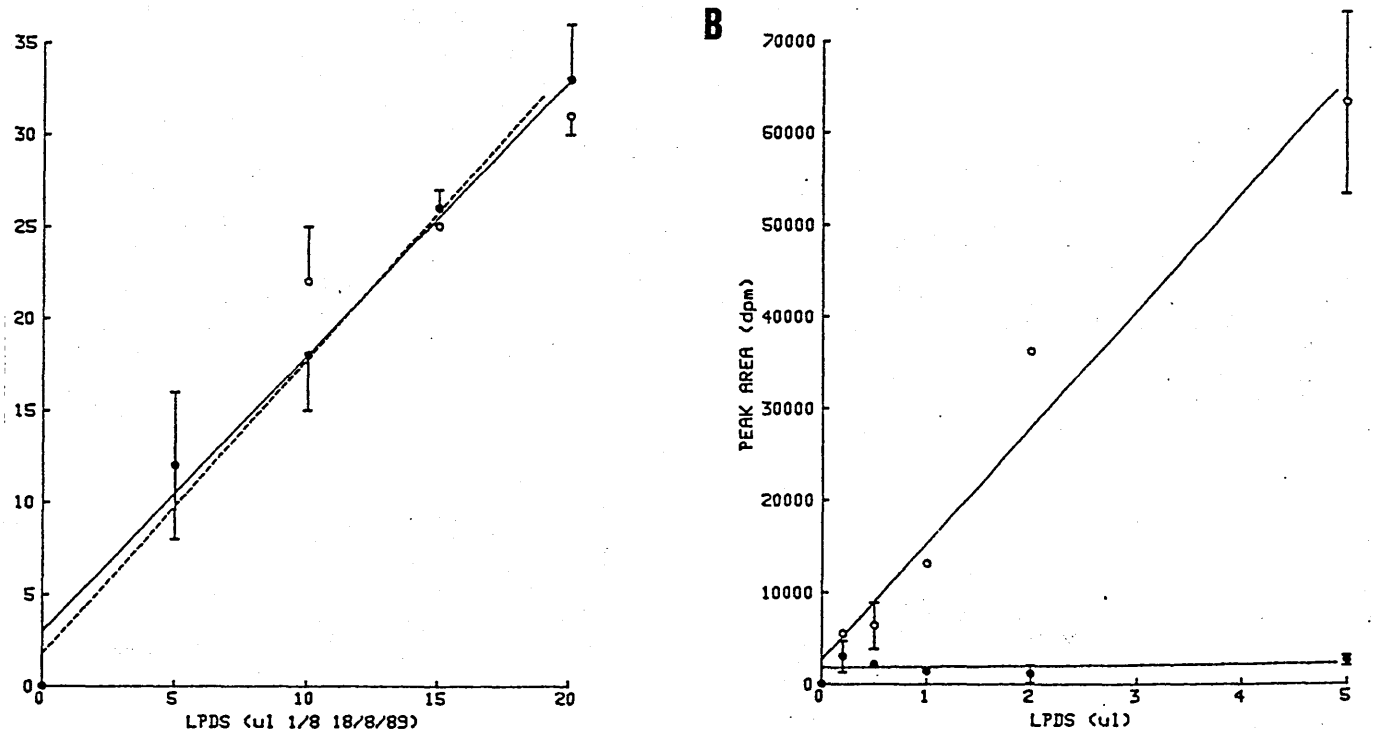


FIGURE 3.18 **EFFECT OF HEAT TREATMENT OF LPDS ON PLASMA CETP AND LCAT ACTIVITY.** LPDS was heated for one hour at 56°*C*. Untreated LPDS was compared with heated LPDS in (A) a CETP assay and (B) an LCAT assay. (○) control, (●) 1 hour at 56°*C*

be separated then interpretation becomes much easier. As CETP is heat stable, if inhibitor is not then inhibitor activity might be removed by heating at a temperature which destroys inhibitor but does not affect CETP. If inhibitor is more heat stable than CETP then it may be possible to inactivate CETP and reveal inhibitory activity. Samples of human LPDP were subjected to various heat treatments as described in Table. 3.7. These samples were then assayed for CETP activity. If the activity measured is the same as the control then it is likely that neither CETP nor inhibitor has been affected. If activity is increased then the inhibitor may have been inactivated, if activity is decreased then CETP may have inactivated. It was found that treatment up to 60°*C* for 30 minutes did not affect activity. Treatment at higher temperatures destroyed all CETP activity.

Treated LPDP was also added to a separate sample of CETP to see if any inhibitory activity could be revealed. If CETP activity is unaffected by treatment then measured CETP activity should increase - this occurred for treatment up to 60°*C* for 30 minutes. If CETP activity is destroyed then the control CETP activity will stay the same if no inhibitory activity is revealed or will decrease if inhibitory activity is revealed. As can be seen from Table. 3.7, no significant inhibitory activity was revealed by any of the treatments. Thus we were unable to separate CETP inhibitor and CETP activity in this manner.

3.3.6 Storage

CETP is stable for at least three months at 4°*C* (Fig. 3.19). An activity profile of the enzyme stored at -20°*C* was carried out as shown in Fig. 3.19. Activity is not greatly decreased over six months. It appears that there is an increase in activity at three months, though this has not been confirmed. The reason for this is not known but could possibly be an effect of the relative stabilities of CETP and inhibitor. It did not matter whether samples were stored as plasma and LPDP prepared after defrosting or were stored directly as LPDP.

TREATMENT	CETP ACTIVITY (% transfer)	INHIBITION OF CONTROL LEVEL BY TREATMENT (%)	ACTIVITY WHEN TREATED LPDS IS ADDED TO CONROL AMOUNT OF CETP (% transfer)	REVEALED INHIBITORY ACTIVITY
BLANK	0		28	none
CONTROL	28	0		none stimulation
42°C O/N	28	0	38	none stimulation
60°C 10 min	28	0	34	none stimulation
60°C 30 min	27	4	39	none stimulation
80°C 10 min	0	100	25	11%
80°C 30 min	0	100	28	0%
100°C 10 min	0	100	27	4%
100°C 30 min	0	100	34	none

TABLE 3.7 **HEAT TREATMENT OF LPDS - CETP ACTIVITY AND CETP INHIBITORY ACTIVITY.** LPDS was subjected to treatment by heat to various degrees. After treatment, the CETP activity of the sample was measured, and also its ability to inhibit a control amount of CETP activity.

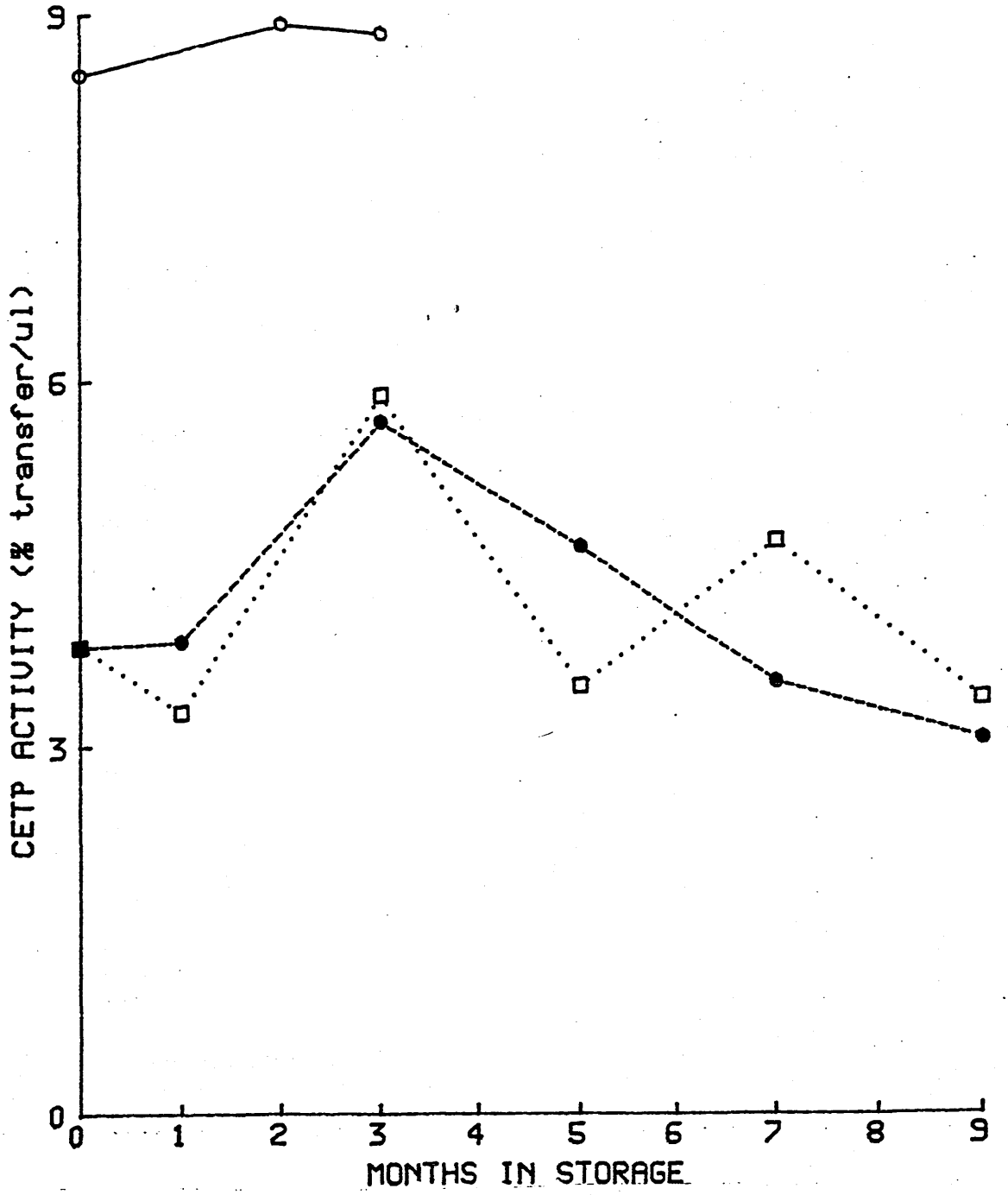


FIGURE 3.19 CETP STORAGE - STABILITY AT 4°C AND -20°C. Human LPDS was stored either at 4°C (○) up to three months or at -20°C as plasma (●) or as LPDP (□) for 9 months. Activity was measured periodically over the time of storage. Where the activity was stored as plasma LPDS was prepared after defrosting by centrifugation at d=1.215g/ml.

CHAPTER IV

CETP PURIFICATION AND ANTIBODY PRODUCTION

1 Introduction

As discussed in the previous chapter, the assay of CETP activity in plasma is complicated. The transfer assay developed measures the sum of CETP and inhibitor protein activity. Thus a measured change in activity could be due to a change in CETP, inhibitor or both. In order to understand this situation more fully it would be beneficial to be able to measure the protein mass of either CETP or inhibitor.

A powerful tool for this kind of measurement would be an antibody, either polyclonal or monoclonal. Antibodies to both human and rabbit CETP were desirable. It was also intended to use rabbit antibodies to test the effect of inhibiting rabbit CETP *in vivo*. Two strategies to production of antibodies were followed:-

a) the information provided from the published cDNA sequence of CETP [Drayna, D. *et al* (1987)] and predicted protein sequence was used to choose a peptide sequence which may be immunogenic. This peptide was used to raise polyclonal antibodies in sheep.

b) partially purified human and rabbit CETP was used to immunise mice in order to obtain monoclonal antibodies.

2 Human CETP Purification

4.2.1 Partial Purification of Human CETP

The partial purification of human CETP was carried out as described in the literature [Pattnaik, N.M. *et al* (1978), Morton, R.E. & Zilversmit, D.B. (1982), Tall, A.R. *et al* (1983) and Jarnagin, A.S. *et al* (1987)]. Human LPDS was used as a starting material. This was adjusted to a high salt concentration (4M NaCl) and loaded onto a phenyl Sepharose column. CETP is bound to the hydrophobic column at this salt concentration. A low salt wash (0.15M NaCl) removes the bulk of the protein and then CETP activity can be eluted with water (Fig. 4.1). It has been reported that this chromatography step removes CETP from its inhibitor protein which remains bound to the column in water [Morton, R.E. & Zilversmit, D.B. (b) (1981), Son, Y-S. C. & Zilversmit, D.B. (1984) and Tollefson, J.H. *et al* (1988)]. However there is one report that CETP inhibitor can be eluted from a phenyl Sepharose column in water [Nishide, T. *et al* (1989)]. Sometimes experimentors have seen a greater than 100%

FIGURE 4.1 **PHENYL SEPHAROSE COLUMN OF HUMAN CETP - WATER ELUTION.** Human LPDS was adjusted to 4M NaCl with solid NaCl and subjected to a 30 minute centrifugation, 35000 rpm Beckman 45Ti rotor, to remove any particulate matter. The supernatant was loaded onto a 200ml phenyl Sepharose column which had previously been equilibrated with 10mM Tris, 2M NaCl, pH8.0 at 4°C. The column was washed at 30ml/hour with 10mM Tris, 0.15M NaCl, pH8.0. Elution was with distilled water, 0.02% sodium azide at 30ml/hour. Fractions (7.5ml) were collected and CETP activity was assayed in 25µl samples of these.

FIGURE 4.2 **CM52 COLUMN OF HUMAN CETP.** Active fractions from the water elution of the phenyl Sepharose were pooled and adjusted to 50mM NaAc, pH 4.5 by addition of 1/19 volume of 1M NaAc pH4.5. A 30 minute centrifugation at 35000rpm removed any particulate matter. The supernatant was loaded onto a 200ml CM52 column preequilibrated with 50mM NaAc, 0.02% sodium azide, 1mg/ml EDTA at 4°C. Activity was eluted at 30ml/hour with a NaCl gradient comprising 300ml 50mM NaAc pH 4.5, 0.02% sodium azide, 1mg/ml EDTA and 300ml of the same buffer but containing 0.2M NaCl. Fractions (7.5ml) were collected in 1ml of 1M TrisHCl pH 7.4 and 25µl samples were assayed for CETP activity. Pooled fractions were concentrated through an Amicon PM10 membrane and dialysed against Tris-saline buffer.

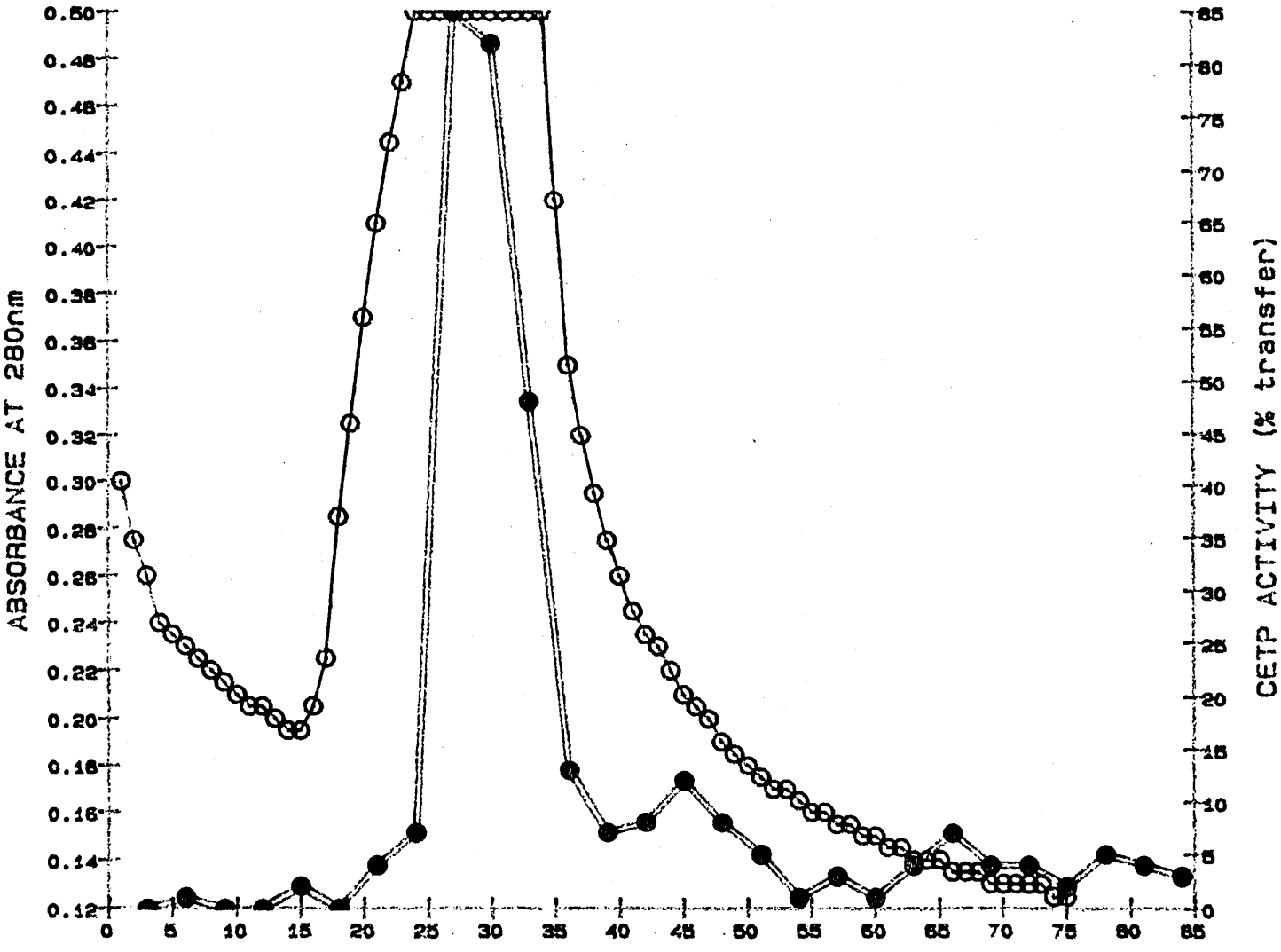

ABSORBANCE AT 280nm
0.50
0.48
0.46
0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
CETP ACTIVITY (% transfer)
PROTEIN
CETP ACTIVITY

Fig 4.1

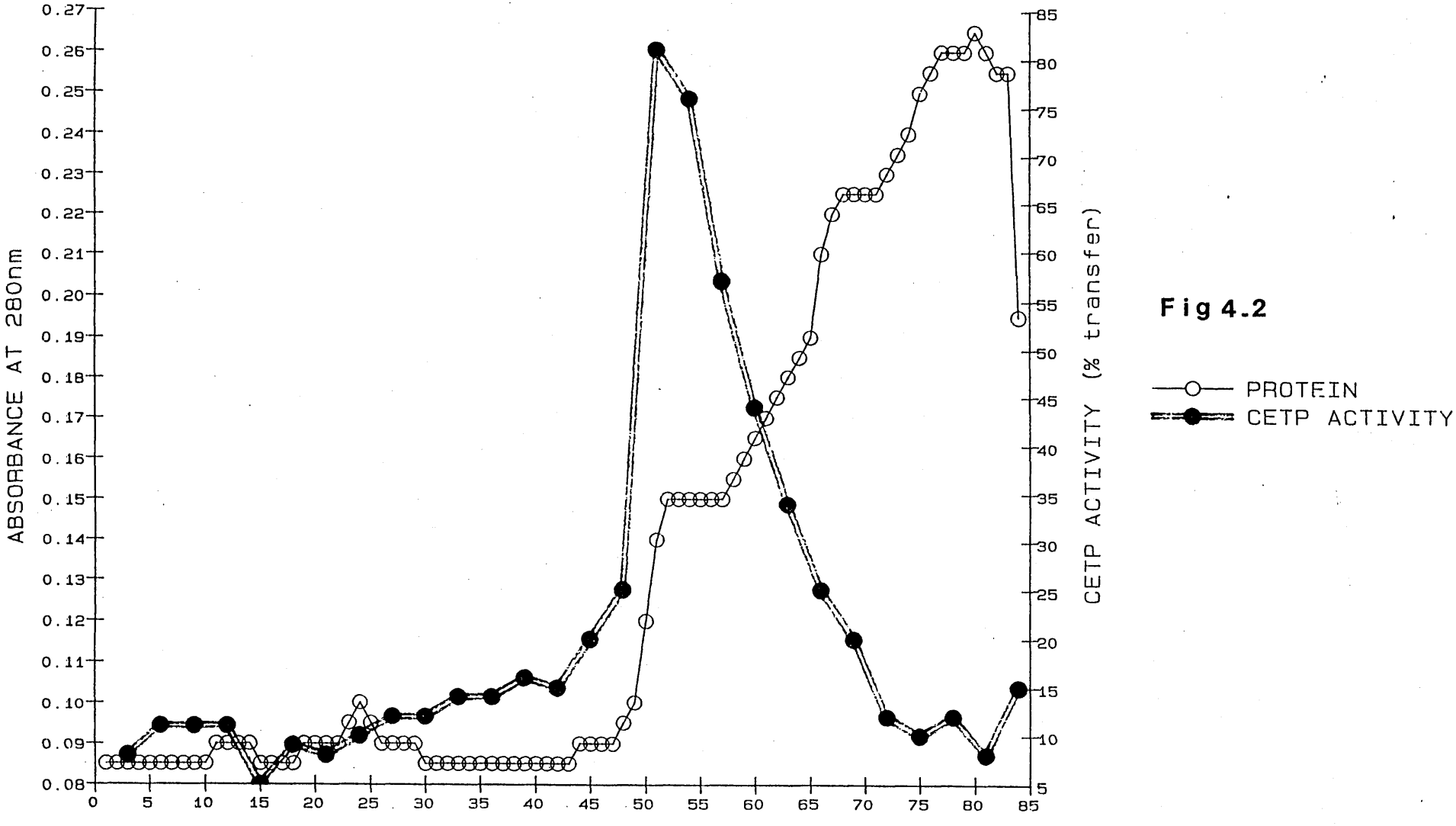

ABSORBANCE AT 280nm
0.27
0.26
0.25
0.24
0.23
0.22
0.21
0.20
0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85
CETP ACTIVITY (% transfer)
85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5
PROTEIN
CETP ACTIVITY

Fig 4.2

	VOLUME (ml)	PROTEIN CONC^{N} (mg/ml)	TRANSFER ACTIVITY (%t/µl)	SPECIFIC ACTIVITY (%t/µl)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (%t)	YIELD (%)	FOLD PURIF^{N}
LPDS	100	32	3.06	0.096	3200	307200		
PHENYL SEPHAROSE	86	1.34	3.65	2.724	115.2	313914	102	28
CM52	12	0.32	8.60	26.88	3.84	103200	33.5	280

TABLE 4.1 PARTIAL PURIFICATION OF HUMAN CETP.

recovery at this stage; this may be due to removal of the inhibitor protein. It was found that in this partial purification there was a slight increase in yield after the phenyl Sepharose column (Table 4.1).

Active fractions from the phenyl Sepharose column were pooled and chromatographed on a CM52 ion exchange column at pH 4.5. Activity was eluted with a 0-200mM NaCl gradient (Fig.4.2). This column separates CETP from LCAT [Pattnaik, N.M. *et al* (1978)]. The partially purified CETP obtained from this procedure was purified 280 fold and obtained in a yield of 34% (Table 4.1). This preparation was stable for at least two months at -20°C. This fraction was referred to as 'post CM52 CETP'.

4.2.2 Further Purification of Human CETP

Various other chromatographic procedures were tried in an attempt to further purify CETP. None of these were particularly successful as the yields tended to be poor due to increased instability of the enzyme as it was purified to a greater extent. Often the extent of purification was not great enough to make the procedure worthwhile. Attempts included batch treatment with hydroxyl apatite [Jarnagin, A.S. *et al* (1987)] where a 6 fold purification was achieved but only 17% recovery in this step. A chromatofocussing column was run as described [Morton, R.E. & Zilversmit, D.B. (1982)] and a profile similar to that published was achieved but it was found that the purified activity was unstable. On a Con A Sepharose column it was found that most activity did not bind and only a small amount of the total loaded on the column eluted with up to 150mM α-methyl-D mannoside. Thus purification was less than one fold. An anti-human albumin column was run to try and remove the large amount of contaminating human serum albumin. Albumin could be removed successfully with this column but recovery of activity was only 6% and the fold purification was less than one indicating that the enzyme was becoming inactivated. All of these purification options were discarded.

4.2.3 Emulsion Binding

During the course of these purification studies, a method was published whereby CETP was purified 55000 fold which included a step where CETP was bound to a synthetic lipid emulsion containing phosphatidyl choline, triolein and oleic acid [Hesler, C.B. *et al* (1987)]. Bound protein was separated from unbound protein by passing down a gel filtration column. An attempt was made to reproduce this purification exploiting the affinity of CETP for lipid particles.

A lipid emulsion of phosphatidyl choline : triolein : oleic acid of composition 0.12:0.80:0.09 was made by sonicating the lipids together and then collecting the lipid emulsion by centrifugation. The lipid was resuspended in 50mM ammonium bicarbonate buffer and then passed down a Sepharose CL-4B gel filtration column. The peak fractions were pooled, concentrated and the lipid composition of the pool was

determined. Emulsion (50mg) was incubated with post CM52 CETP (8mg) for 15 minutes at 37°C under nitrogen and then chromatographed on a Sepharose CL-4B gel filtration column. The column profile is shown in Fig. 4.3B.

As can be seen from Fig. 4.3A, when CETP alone is passed down a gel filtration column, the unbound post CM52 CETP elutes at fraction 30 (i.e. column volume of 80ml) slightly later than the main protein peak. When post CM52 CETP is first bound to the lipid emulsion and then run on a Sepharose CL-4B gel filtration column, CETP activity is separated into two main peaks (Fig. 4.3B). One elutes at fraction 28 presumably as unbound CETP. The other peak elutes at fraction 18 just after the milky lipid peak at fraction 16. Thus it appears that a proportion of the CETP activity does bind to the lipid emulsion but a larger proportion is unbound. Therefore the emulsion used here was not binding optimal amounts of CETP.

In order to improve the lipid binding of CETP another type of emulsion was used. Intralipid 10% is a commercially available lipid emulsion of composition soyabean oil (10%) : fractionated egg phospholipid (1.2%) : glycerol (2.25%) (w/v). Post CM52 CETP (8mg) was incubated with Intralipid (50mg) for 30 minutes at 37°C under nitrogen. This was then passed down a Sepharose CL-4B gel filtration column. As can be seen from Fig. 4.3C, CETP activity elutes as one peak at fraction 15 (40ml). No activity elutes at the position of unbound CETP.

Fig. 4.4 shows an SDS polyacrylamide gel of the fractions across one such column. Serum albumin occurs across all the fractions but a band running more slowly at the position which might be expected for CETP appears in fractions 20 to 22. The average molecular weight calculated from SDS polyacrylamide gels was 76300 +/- 3700, n=5.

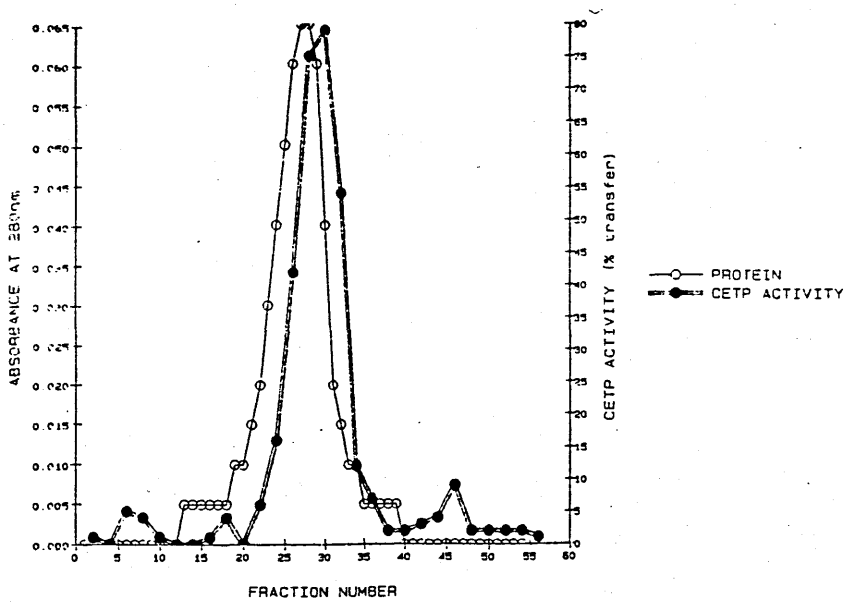
In order to check that this band was indeed CETP, a Western blot was carried out using a mouse anti-human CETP IgM monoclonal antibody (a gift from Hans Dieplinger, Innsbruck). Fig. 4.5 shows the SDS polyacrylamide gel of human serum albumin and CETP bound to Intralipid. This was electroblotted onto nitrocellulose and then immunochemical detection was carried out using the monoclonal as first antibody and a goat anti-mouse IgM conjugated to horse radish peroxidase as second antibody. The monoclonal picks out the band which runs slower than the 68000 serum albumin band which had been tentatively described as CETP. Thus this antibody identified the protein that had been purified as human CETP.

Attempts were made to remove the protein from the lipid complex. Ethanol:diethyl ether delipidation resulted in complete loss of activity. The solvents may affect the enzyme, but this technique has previously been successful [Hesler, C.B. *et al* (1987)]. Another agent, used for delipidating plasma, Cab-O-Sil, was used to try and delipidate the CETP intralipid complex but it was found that Cab-O-Sil bound CETP as well as the lipid. Since monoclonal antibodies do not require a pure antigen it was decided to use the CETP still bound to Intralipid as an immunogen.

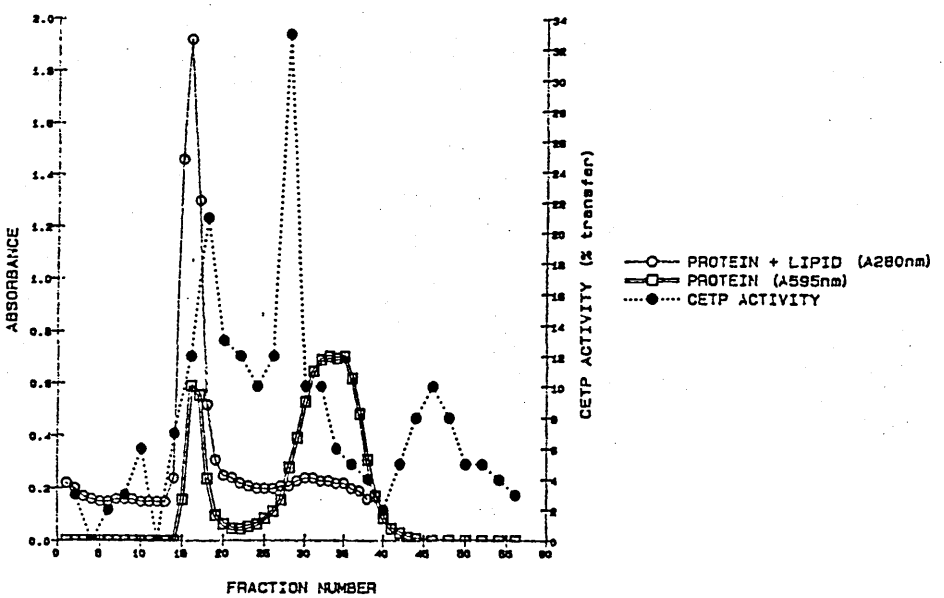
FIGURE 4.3 **SEPHAROSE CL-4B COLUMN - HUMAN CETP, FREE AND BOUND TO EMULSION OR INTRALIPID.** (A) Human CETP (3.8mg) was loaded onto a 100ml CL-4B column and eluted with Tris-saline buffer at 11ml/hour. Fractions (2.75ml) were assayed for CETP activity. (B) Emulsion, 0.12:0.80:0.08 PC:TO:OA (50mg) was incubated with human post CM52 human CETP (8mg) for 15 minutes at 37°C under nitrogen. This was loaded onto a 100ml Sepharose CL-4B column equilibrated with 50mM ammonium bicarbonate, 0.5mM EDTA, pH 8.5 and eluted at 20ml/hour with the same buffer. Fractions (5ml) were collected and assayed for CETP activity and protein content using a Bradford assay. (C) Intralipid (50mg) and human post CM52 CETP (8mg) were incubated together for 30 minutes at 37°C under nitrogen. This was loaded onto a 100ml Sepharose CL-4B column and eluted at 11ml/hour in 50mM ammonium bicarbonate buffer, 0.5mM EDTA pH 8.5. Fractions (2.75ml) were collected and assayed for CETP activity and protein content by Bradford assay.

Fig 4.3

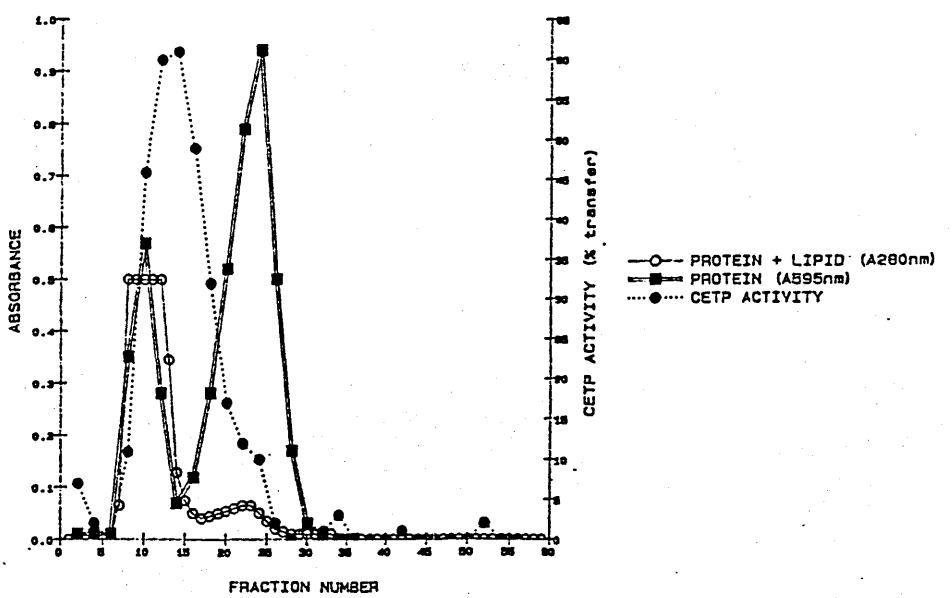
A


ABSORBANCE AT 280nm
CETP ACTIVITY (% transfer)
FRACTION NUMBER
PROTEIN
CETP ACTIVITY

B


ABSORBANCE
CETP ACTIVITY (% transfer)
FRACTION NUMBER
PROTEIN + LIPID (A280nm)
PROTEIN (A595nm)
CETP ACTIVITY

C


ABSORBANCE
CETP ACTIVITY (% transfer)
FRACTION NUMBER
PROTEIN + LIPID (A280nm)
PROTEIN (A595nm)
CETP ACTIVITY

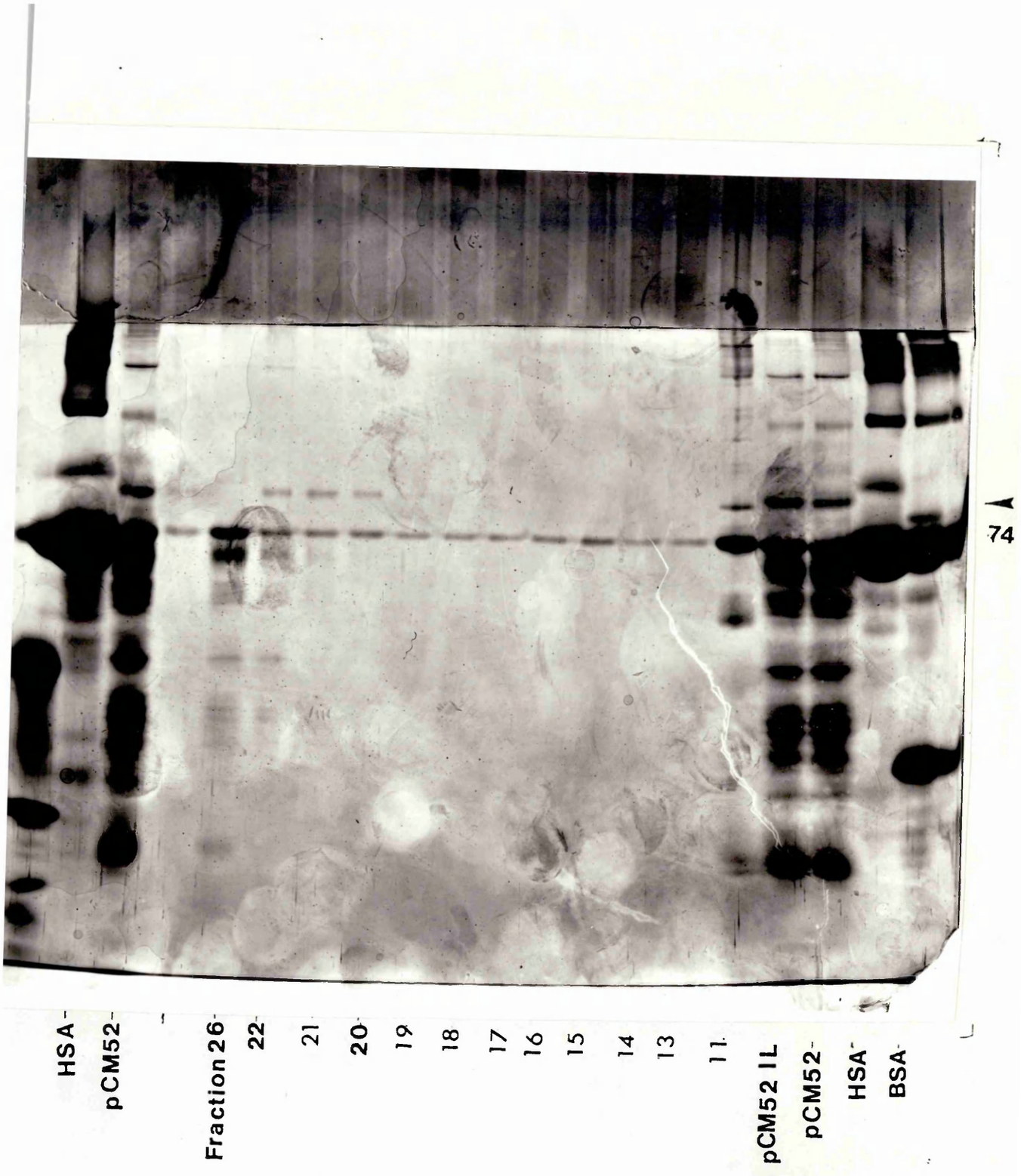


FIGURE 4.4 **SDS POLYACRYLAMIDE GEL ELECTROPHORESIS - SEPHAROSE CL-4B FRACTIONS, HUMAN CETP BOUND TO INTRALIPID.** Fractions were loaded in sample buffer containing 5% 2-mercaptoethanol and saturated with urea, onto a 10% polyacrylamide gel. The gel was run overnight at 30V and then silver stained.

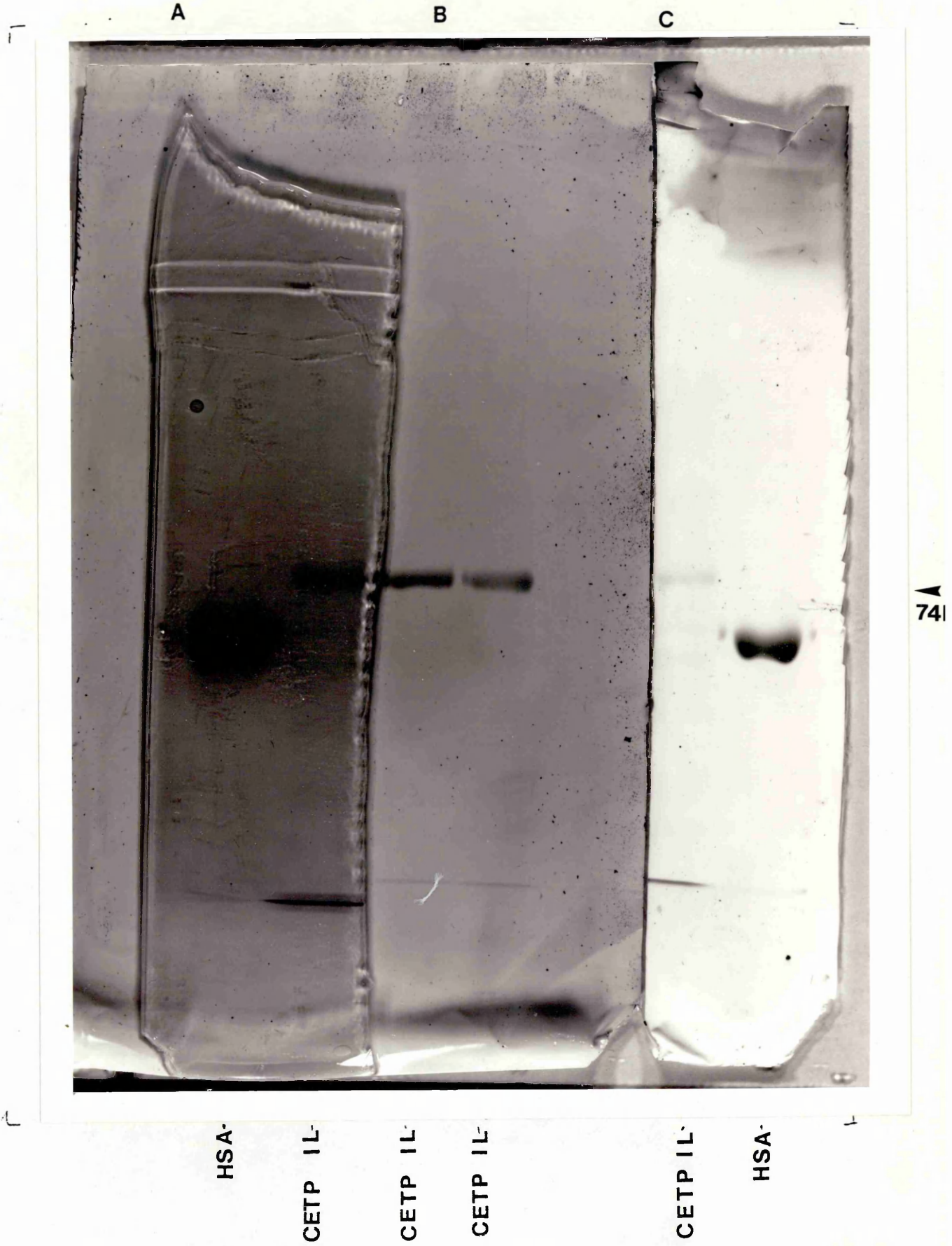


FIGURE 4.5 WESTERN BLOT OF HUMAN CETP. A preparation of human CETP bound to Intralipid was run on a 7.5% SDS polyacrylamide gel. Part of the gel was stained with Coomassie blue (A). The rest of the gel was then blotted onto 0.2 micron nitrocellulose filter at 250mA for 3 hours. The nitrocellulose filter was then stained with Ponceau red (C) or blocked with Tris buffered saline, 0.02% TWEEN 20, 5% BSA (B). The first antibody was a 1/50 dilution of a mouse anti-human CETP IgM monoclonal (gift from H. Dieplinger). The second antibody was 1/500 dilution of goat anti-mouse IgM conjugated to horse radish peroxidase. The stain was developed using 4-chloro-1-napthol and hydrogen peroxide. The reaction was stopped with sodium azide.

3 Rabbit CETP Purification

4.3.1 Partial Purification

A similar purification procedure to that used for human CETP was carried out for rabbit CETP. Again LPDS was used as a starting material. The behaviour of the CETP activity on the phenyl Sepharose and CM52 columns differed for the rabbit material. For the human enzyme, no activity was eluted from the phenyl Sepharose column by the low salt wash and most of the activity eluted with water. However for the rabbit enzyme some activity was eluted with 0.15M NaCl - peak A (Fig. 4.6A) and a further peak of activity was eluted with water - peak B (Fig.4.6B). These peaks of activity were pooled separately and loaded onto CM52 columns. When peak A was chromatographed on a CM52 column the activity eluted at fractions 13-14 (Fig. 4.7A). However when peak B was chromatographed on a CM52 column the peak of activity eluted at fraction 66 (Fig. 4.7B). To try and ascertain whether these activities were different, the activity peaks from the two CM52 columns were pooled and rechromatographed on a CM52 column to see if the two activities could still be distinguished. Two peaks were resolved on this column at fractions 18 and 57 (Fig. 4.7C) which look like they may represent the peaks separated earlier. However this result is not conclusive since the peaks do not coincide exactly with the position where they ran before and also the first peak at fraction 18 is represented only by one measurement of CETP activity. There was insufficient time to study this anomaly further. If there are two distinct peaks of activity on the CM52 column then this may indicate that CETP can exist in two forms which differ chromatographically or that rabbit plasma expresses two CETP activities. When peak A and peak B from the phenyl Sepharose column were run on an SDS polyacrylamide gel the proteins in the two pools were different but the fractions were insufficiently purified to discern which bands may be responsible for one or other activity (Fig. 4.8). The CETP activity which behaved similarly to human CETP was recovered in 60% yield but only 120 fold purification.

4.3.2 Emulsion Binding

In order to make an antigen for antibody production, the rabbit CETP activity which behaved in a similar manner to the human activity was bound to Intralipid and passed down a Sepharose CL-4B gel filtration column. The activity bound to the trailing edge of the lipid peak at about one half the column volume (Fig. 4.9B) though the activity peak was much broader than was the case for the human enzyme. Unbound rabbit CETP elutes at the column volume (Fig. 4.9A). An SDS polyacrylamide gel of the column fractions showed the appearance of a slower running band in fractions 14-16 (Fig. 4.10). The molecular weight of this band calculated from five gels was 76200 +/- 1200.

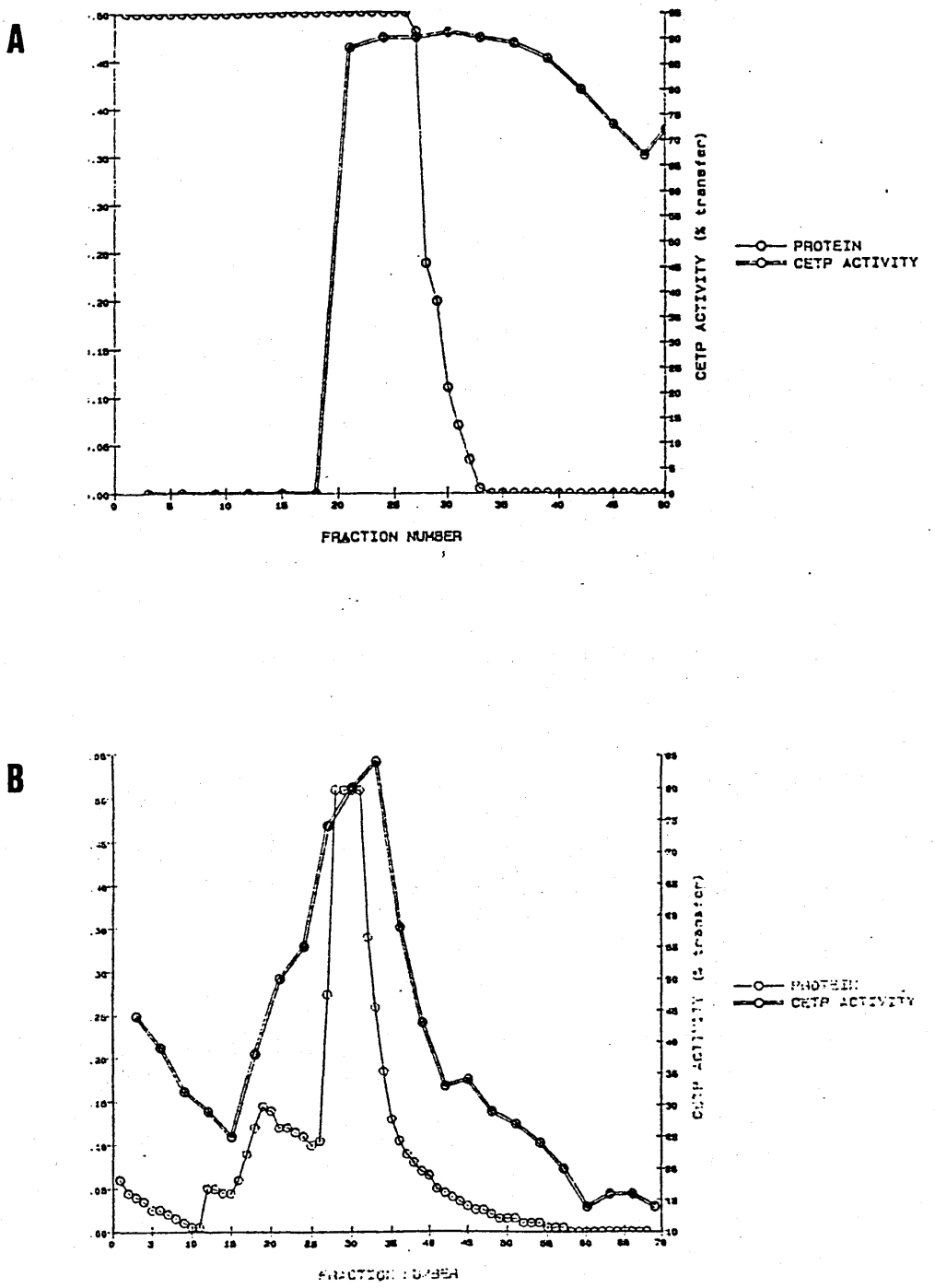


FIGURE 4.6 **RABBIT CETP PURIFICATION - PHENYL SEPHAROSE COLUMN.** Rabbit LPDS was made to 4M NaCl and loaded onto a 200ml phenyl Sepharose column equilibrated with 10mm Tris, 2M NaCl, pH 8.0. Elution was carried out at 25ml/hour collecting 8ml fractions. A) Elution with 10mM Tris, 0.15M NaCl, pH 8.0. B) Elution with distilled water, 0.02% sodium azide. Fractions were assayed for CETP activity.

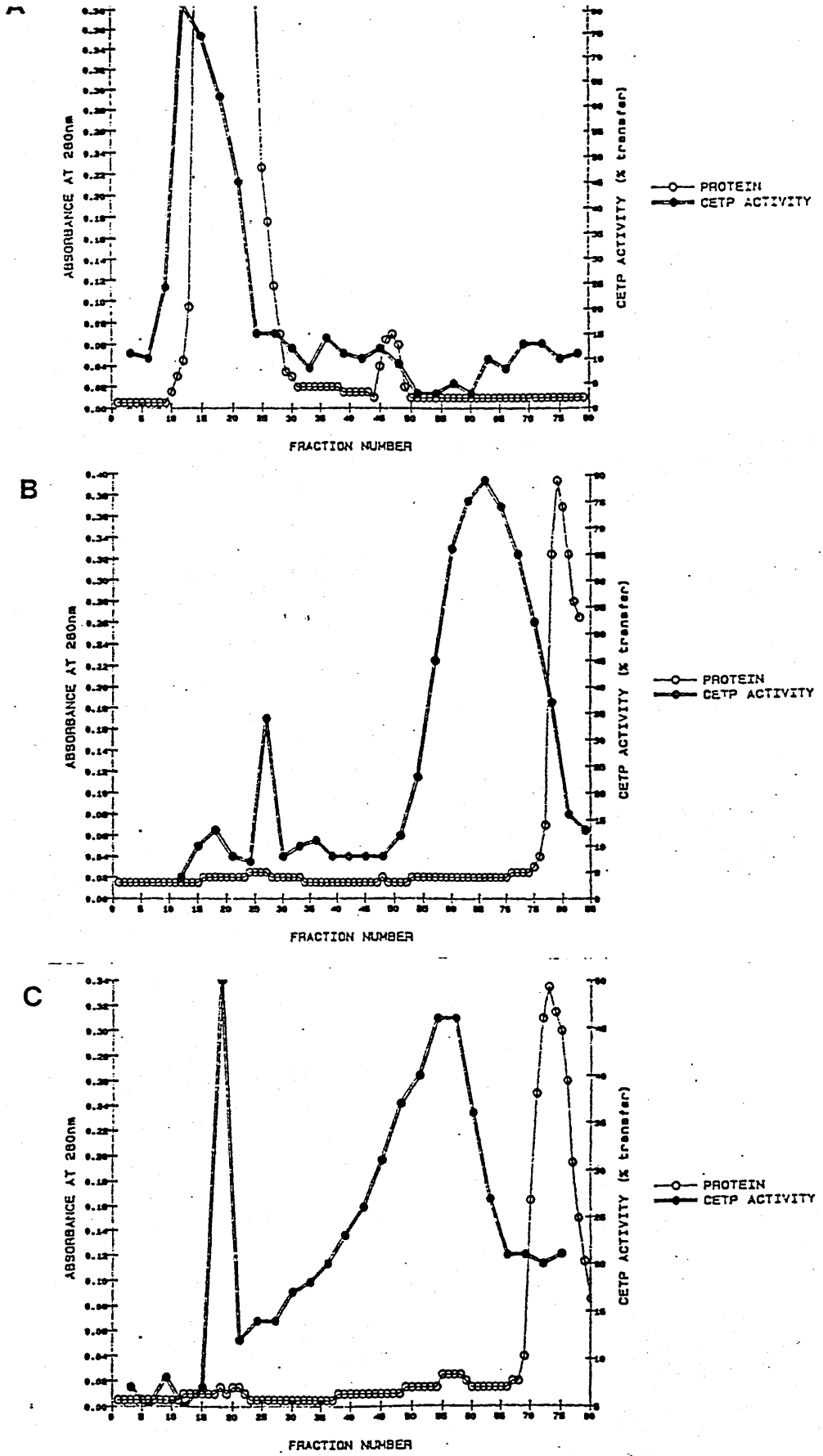


FIGURE 4.7 **RABBIT CETP PURIFICATION - CM52 COLUMN.** Samples of A) pooled activity from the 0.15M salt wash of the phenyl Sepharose column B) pooled activity from the water wash of the phenyl Sepharose column and C) pooled activity peaks from the two CM52 columns A and B, were adjusted to 50mM NaAc, 0.02% sodium azide, 1mg/ml EDTA, pH 4.5 and loaded onto a 200ml CM52 column prequilibrated with 50mM NaAc, 0.02% sodium azide, 1mg/ml EDTA pH 4.5. Columns were eluted at 30ml/hour with a 0-200mM NaCl gradient in acetate buffer. Fractions (7.5ml) were collected in 1M TrisHCl pH 7.4 and assayed for CETP activity.

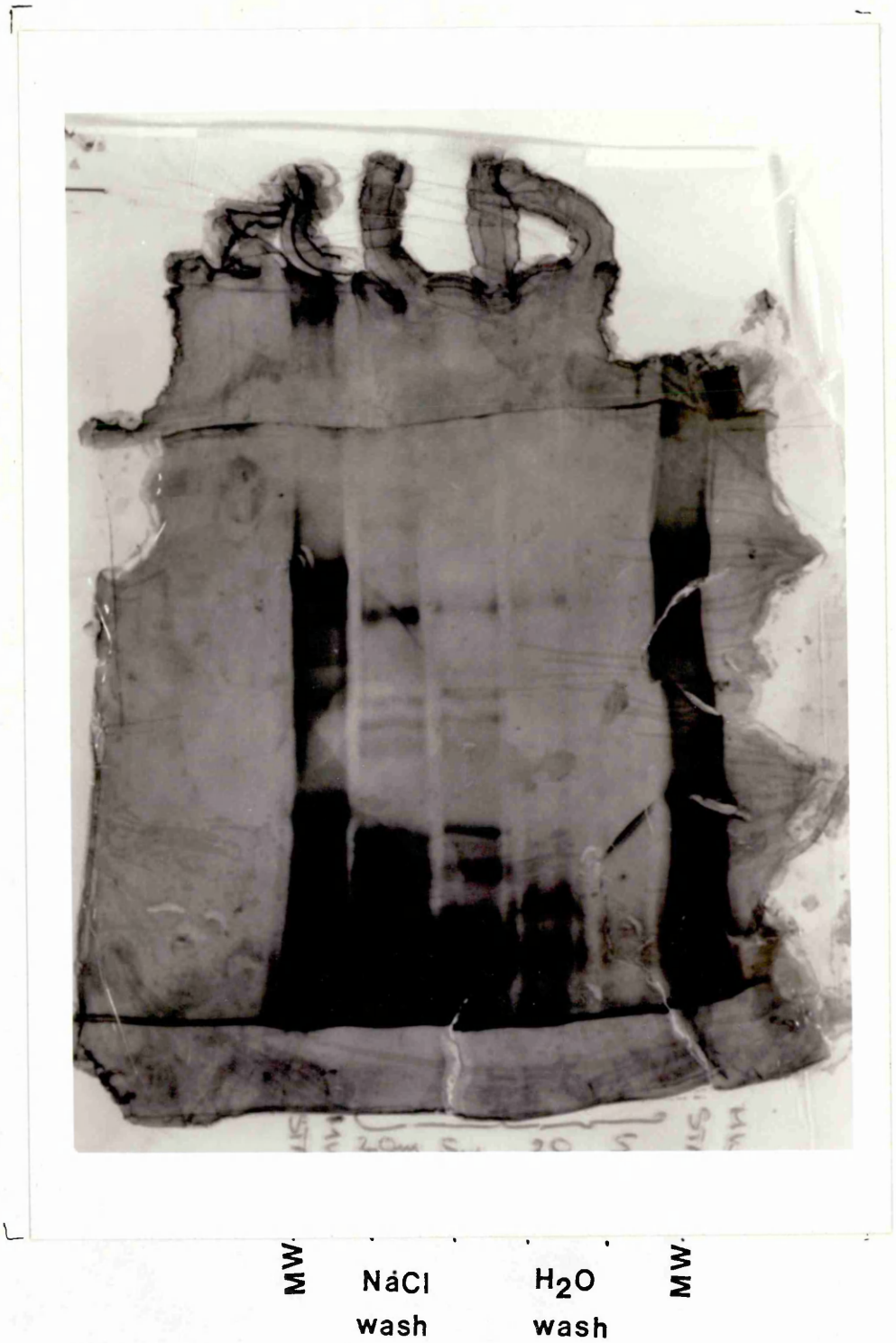


FIGURE 4.8 **GEL ELECTROPHORESIS OF CM52 PEAKS.** Pooled peaks from the CM52 column loaded with the low salt wash (peak A) and the water wash (peak B) of the phenyl Sepharose column were electrophoresed on a 7.5% SDS polyacrylamide gel and silver stained.

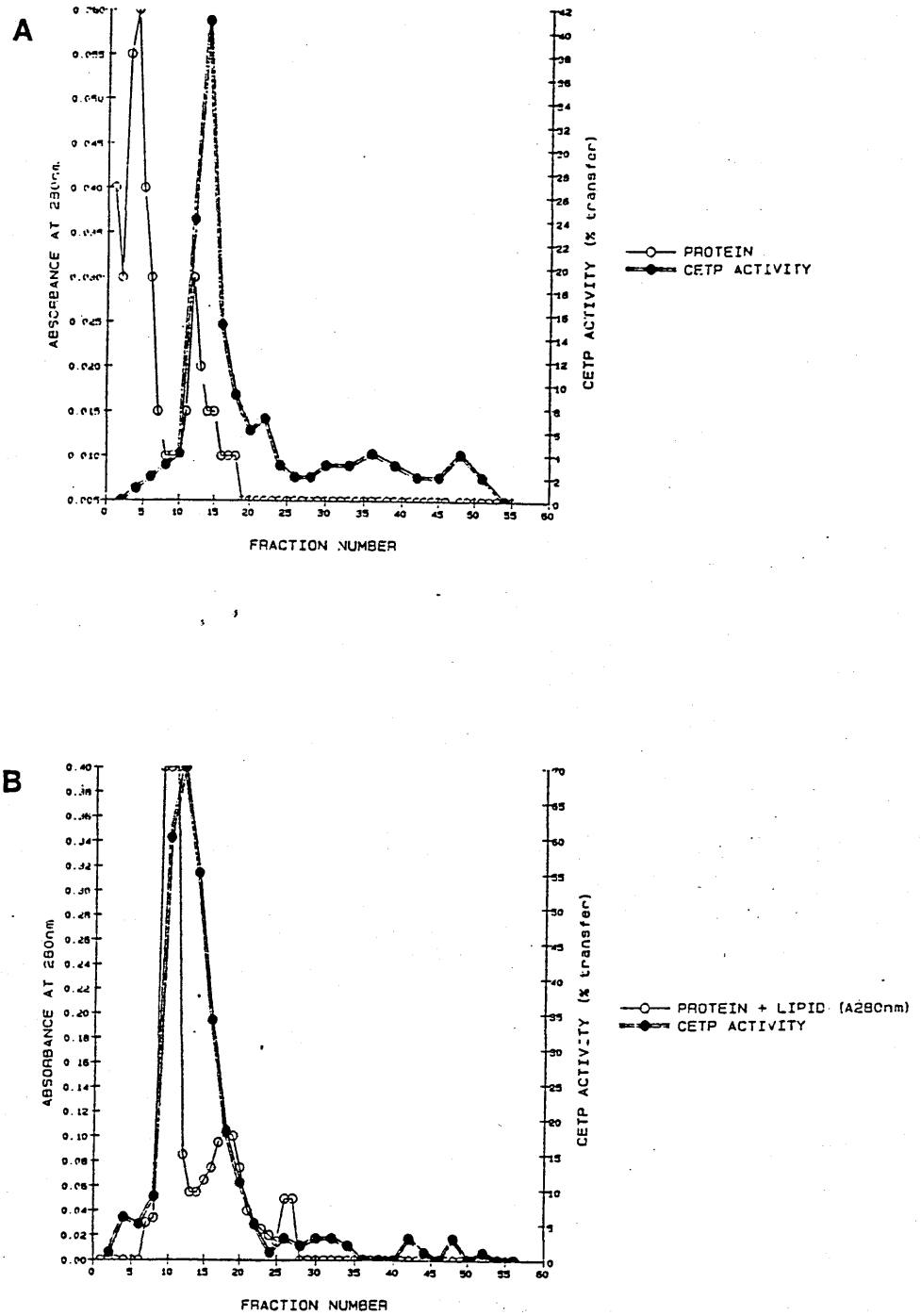


FIGURE 4.9 **CL-4B GEL FILTRATION COLUMNS OF RABBIT CETP.** Rabbit CETP (A) or rabbit CETP bound to Intralipid (B) were loaded onto a 30ml (A) or 200ml (B) Sepharose CL-4B column and eluted at 11ml/hour (A) or 30ml/hour (B) with 50mM ammonium bicarbonate, 0.5mM EDTA pH 8.5. Fractions, 2.75ml (A) or 7.5ml (B)were collected and assayed for CETP activity.

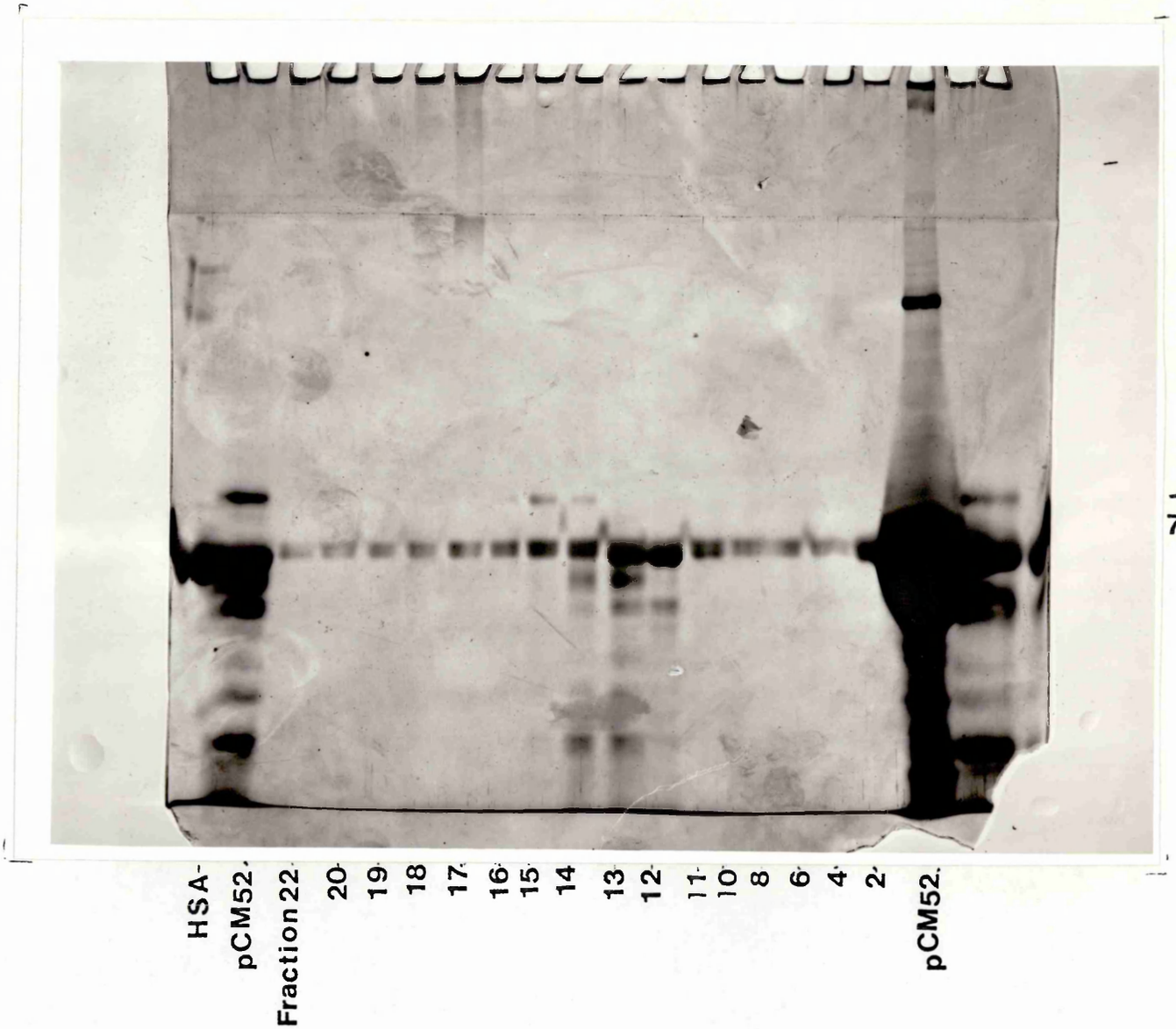


FIGURE 4.10 **GEL ELECTROPHORESIS OF RABBIT CETP BOUND TO INTRALIPID COLUMN FRACTIONS FROM THE CL-4B COLUMN.** Fractions from the Sepharose CL-4B column of rabbit CETP bound to Intralipid were run on a 7.5% SDS polyacrylamide gel and silver stained.

4 Antibody Production

4.4.1 Antibodies to CETP Peptide Raised in Sheep

One strategy to make antibodies was to choose a peptide from the CETP sequence which may be particularly immunogenic. This peptide can then be synthesised and used to raise antibodies which will hopefully react against the whole protein. Using the published amino-acid sequence which was predicted from the cDNA nucleotide sequence [Drayna, D. *et al* (1987)], a hydrophilicity profile and predicted secondary structure was ascertained. The hydrophilicity plot was drawn by the 'Predict' program (Peter Murray-Rust, Glaxo Group Research). Secondary structure was predicted by Chou and Fasman and Robson plots (Appendix 1). Also the consensus secondary structure from eight predictions of secondary structure was plotted (Fig. 4.11) (program by Elios Eliopolous, Leeds). From these plots several stretches of peptide sequence were chosen which had a predicted β-turn and were hydrophilic. These peptide properties were sought since it is likely that a part of the molecule with those properties would be external to the protein molecule and exposed. An exposed part of the molecule is more likely to be immunogenic than one which is internal. One of these peptides was finally selected by a chemist, Dr Peter Seale (Glaxo Group Research Ltd, Greenford) and synthesised with a cysteine residue added to the N terminus to aid conjugation to a protein carrier molecule. The sequence chosen was from residue 351 to 360 and is shown in Fig. 4.12. The equivalent peptide from the rabbit protein is also shown. There were four changes in this sequence for the rabbit. One of these changed an uncharged polar residue to a basic one (Gln - Arg) and another from a basic residue to an acidic one (His - Glu). It was not known whether this would affect cross reactivity if an antibody were obtained.

To prepare the antigen the peptide was conjugated to keyhole limpet haemocyanin (KLH). Conjugated peptide was emulsified with oil and Freund's complete adjuvant and used (18μg) to inject one Black Face sheep. Immunisation was by four subcutaneous injections; two subscapular and two on the haunches. The animal was boosted a month later with the same amount of antigen but in incomplete Freunds adjuvant and the first sample bleed taken two weeks after the boost. Sheep serum showed 26% inhibition of both rabbit post CM52 CETP and human LPDS at a dilution of 1/10, over and above any inhibitory activity of sheep serum itself. An ELISA was carried out to test the binding of the antiserum to CETP peptide, KLH and human post CM52 CETP (Fig. 4.13). Non-immune serum showed negligible binding to any of these three antigens. The antiserum showed good binding activity against both the CETP peptide and KLH alone. However the antiserum did not recognise human post CM52 CETP. Thus antibodies were successfully raised against the immunogens but these antibodies were unable to recognise the native protein in the post CM52 preparation. This result illustrates a pitfall of using peptides to raise antibodies. The peptide selected does not necessarily take up the conformation that it would in the native protein and therefore antibodies against the peptide conjugate do not necessarily

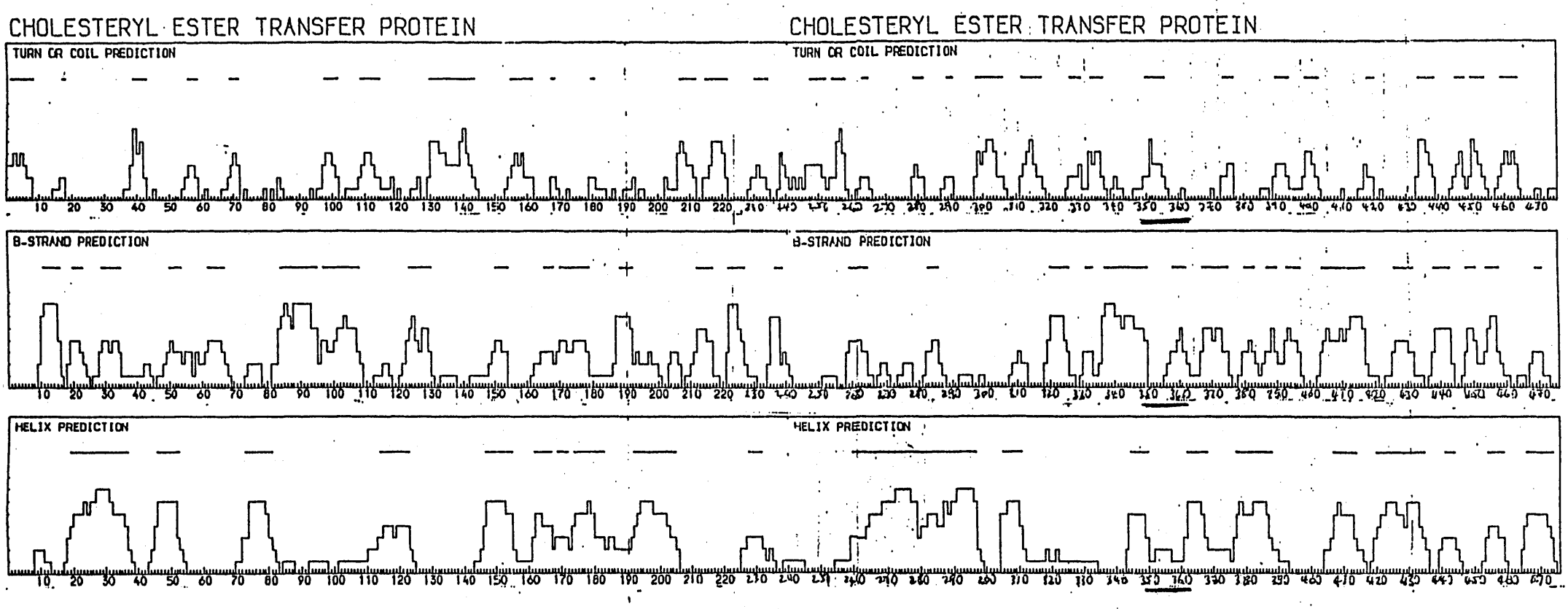


FIGURE 4.11 **CONSENSUS SECONDARY STRUCTURE PREDICTIONS FOR HUMAN CETP.** Eight models for predicting secondary structure; the Burgess, Dufton, Fasman, Garnier, Kabat, Lim, McLauchlan and Nagano models; were plotted for human CETP using a computer program (Elios Eliopolous, Leeds). The consensus agreement between these models is shown with frequency of agreeing predictions for each amino-acid residue.

A) HUMAN Cys-Pro-Arg-Pro-Asp-Gln-Gln-His-Ser-Val-Ala

351 360

B) RABBIT Pro-Arg-Pro-Asp-Gly-Arg-Glu-Ala-Val-Ala

FIGURE 4.12 SEQUENCE OF THE PEPTIDE CHOSEN FOR PRODUCTION OF POLYCLONAL ANTIBODIES. The peptide chosen comprises amino-acid residues 351 to 360. A cysteine residue was put on the N terminal end to allow conjugation to a carrier protein. A) The human sequence. B) The rabbit sequence.

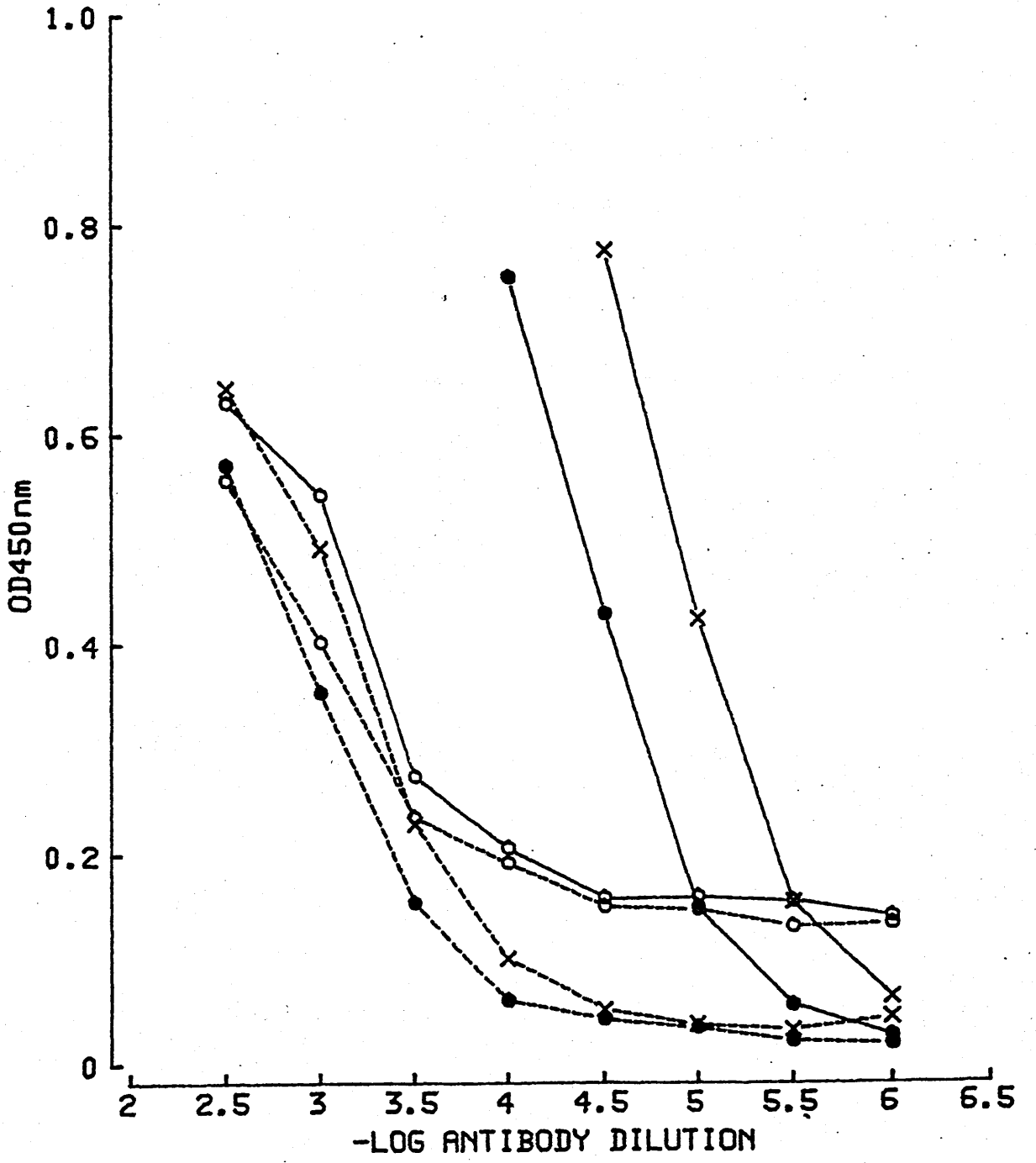


FIGURE 4.13 ELISA OF SHEEP IMMUNE SERUM. A sheep was immunised with the CETP-peptide conjugated to KLH. The immune serum was tested in an ELISA for its activity against the CETP peptide (●), KLH (×) and human post CM52 CETP (○). Both immune (—) and non-immune (– –) sera were tested. Plates were coated with antigen at 1μg/ml. The second antiserum was a donkey anti-sheep IgG conjugated to horse radish peroxidase at 1/1000 dilution.

react against the native protein. It is possible that the antibodies raised may recognise the denatured protein and thus a Western blot was carried out to see whether any reaction could be seen with the CETP band. This type of analysis showed that there was a strong specific reaction with KLH but also a non-specific staining showing many other bands in the human post CM52, rabbit post CM52 and the human serum albumin tracks (Fig. 4.14). Nothing reacted at the expected molecular weight for CETP. This reaction could not be used to detect the amount of CETP in impure preparations. The work with this antiserum was suspended since it did not prove to be a useful tool.

4.4.2 Monoclonal Antibodies to Rabbit CETP

Two batches of mice were immunised with two different preparations of rabbit CETP. The first was partially purified CETP to the post CM52 column stage. The second preparation used CETP bound to Intralipid. In order to use the specific CETP band for immunisation, the CETP bound to Intralipid was run on an SDS polyacrylamide gel and then blotted onto a nitrocellulose membrane. The protein bands could be visualised by staining with Ponceau red. After cutting the appropriate band from the nitrocellulose and destaining in phosphate buffered saline, the nitrocellulose with bound CETP could be dissolved in DMSO and used to immunise the mice (10μg). Mice tail bleeds were tested two months later and it was found that those immunised with the rabbit post CM52 preparation showed best inhibitory activity. These mice were boosted with 10μg of post CM52 CETP before spleen cells from two mice were fused with NS-1 cells. Two months after fusion the colonies had grown such that the supernatants could be tested. The cultures were cloned three times to a dilution of 0.3 cells per well. After each cloning stage cell supernatants were tested for inhibition of CE exchange in a CETP assay, using rabbit post CM52 as an enzyme source, and also of TG exchange in case these activities could be inhibited separately. At the last dilution stage, three positives (10.8, 10.15 and 10.16) were found to inhibit CETP activity by between 50 and 60%. None of the positives found in these screens inhibited human CETP. Large cultures of these were grown such that they could be injected into the peritoneal cavity of mice to produce larger amounts of antibody. The production of antibodies was slow in these mice so another batch of mice were innoculated with a greater number of cells. Ascites from clone 10.15 showed dose dependent inhibition of CETP activity (Fig. 4.15) but only to 20% inhibition. A partially purified preparation (by ammonium sulphate precipitation and Sephadex G200 column) of this ascites showed no inhibition. A second batch of the 10.15 clone was grown up to try and increase the amount of of antibodies. However the second batch of 10.15 ascites showed no activity at all. It is possible that the clone had become overrun with non-producer cells. Fused cells can stop production of antibodies for no apparent reason or the instability of these cells can result in chromosones being lost. It was decided not to proceed with development of these antibodies further

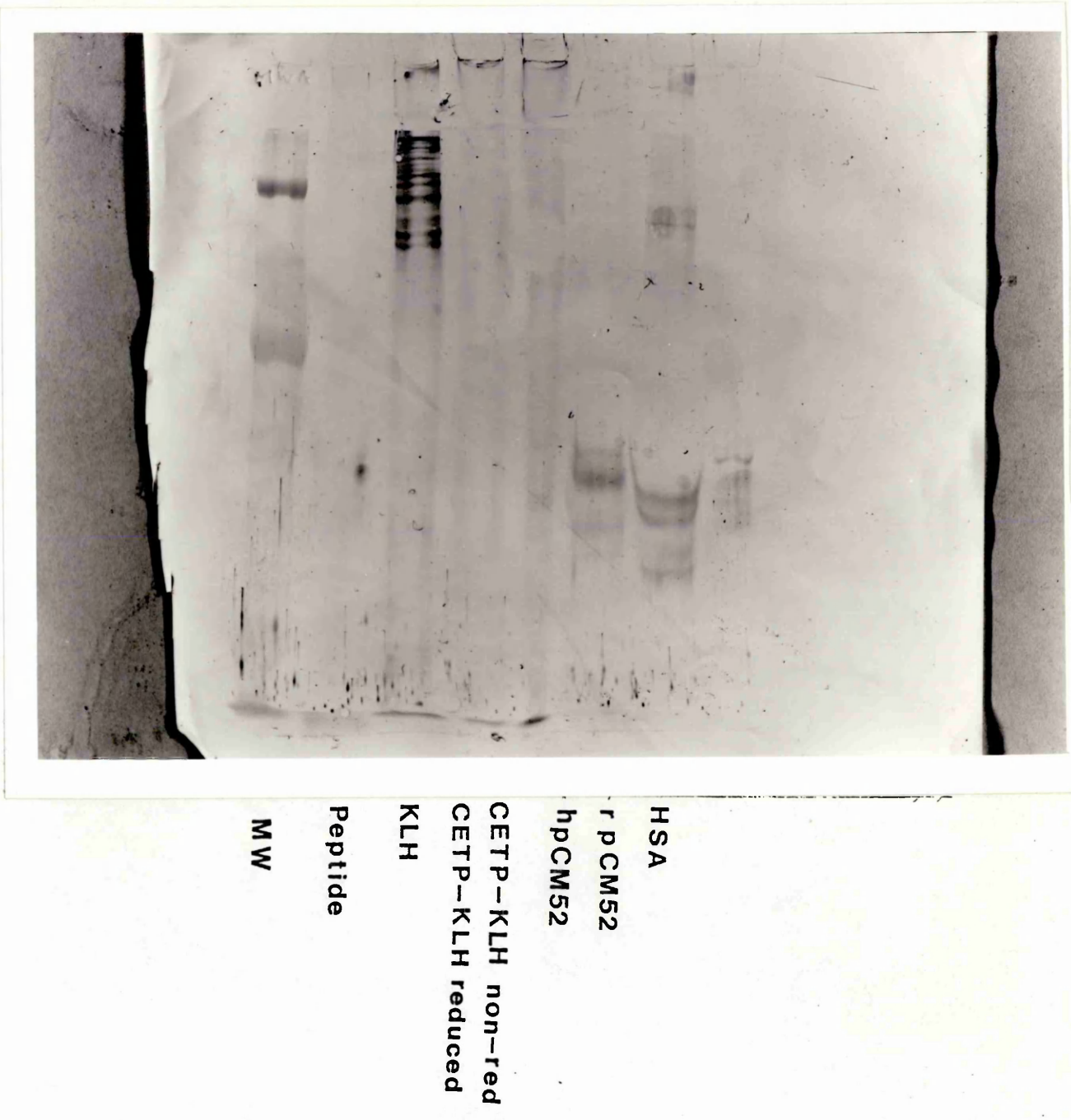


FIGURE 4.14 **WESTERN BLOT OF HUMAN POST CM52 CETP USING SHEEP IMMUNE SERUM.** Samples were run on a 7.5% SDS polyacrylamide gel and then electroblotted onto a 0.2 micron nitrocellulose filter. For immunodetection, the filter was blocked in 5% BSA and then exposed to a 1/100 dilution of sheep immune serum. The second antibody used was a 1/1000 dilution of donkey anti-sheep IgG conjugated to horse radish peroxidase.

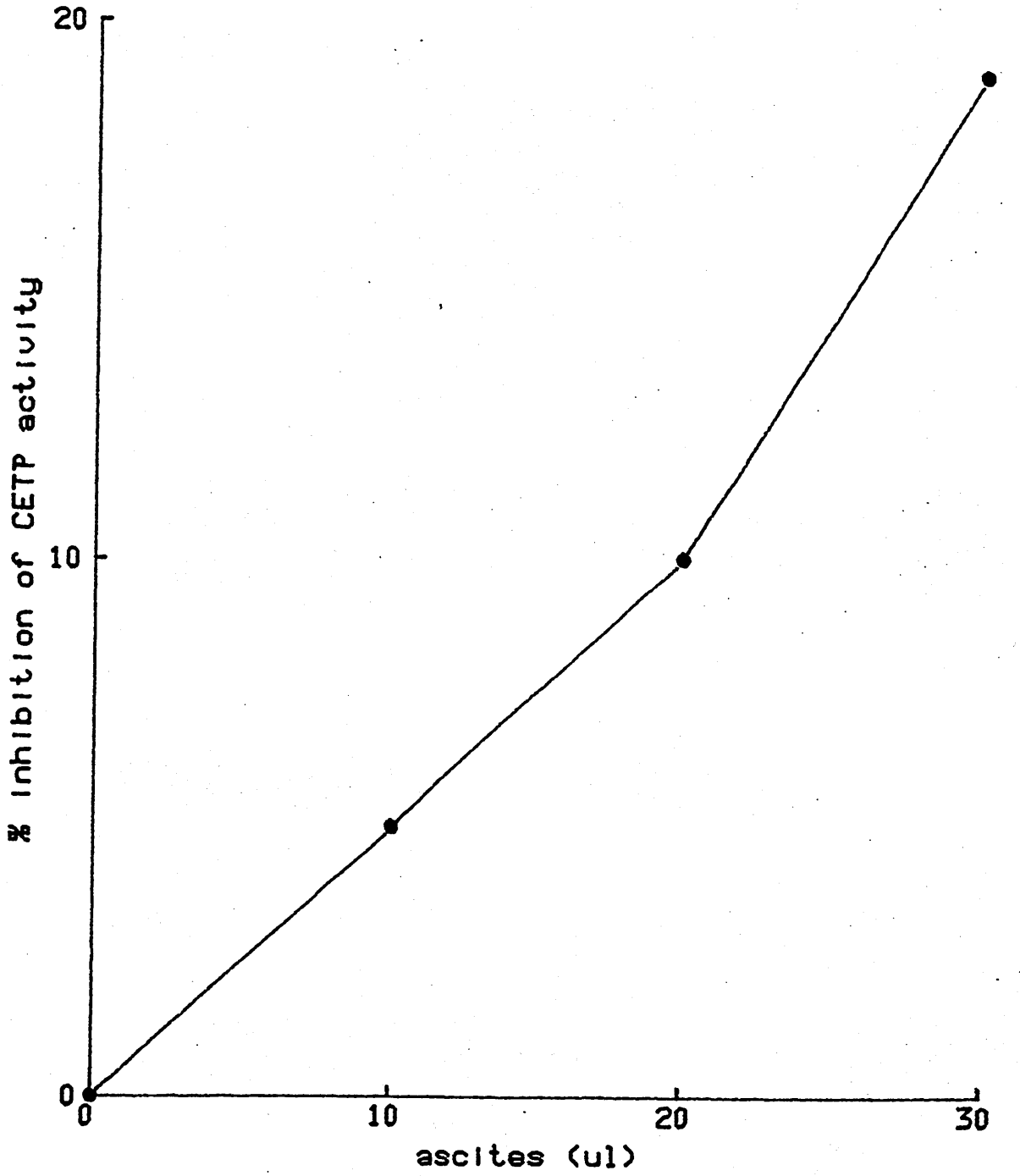


FIGURE 4.15 CETP ASSAY FOR THE INHIBITORY EFFECTS OF CLONE 10.15 ANTI-RABBIT CETP SUPERNATANT. A mouse was inocculated with cells from the 10.15 clone. Ascites were tapped after 2.5 weeks. The ascites was added to a CETP assay containing a control amount of rabbit post CM52 CETP.

since events in the literature had superceded the requirement to make antibodies to rabbit CETP in order to inhibit rabbit CETP activity *in vivo* [Whitlock, M.E. *et al* (1989), Abbey, M. & Calvert, G.D. (1989)].

4.4.3 Monoclonal Antibodies to Human CETP

As with the immunisation of mice for rabbit CETP, the mice for monoclonal antibodies to human CETP were immunised with CETP bound to nitrocellulose prepared from the CETP bound to Intralipid preparation, obtained from Western blotting of a polyacrylamide gel, and dissolved in DMSO. Mice were also immunised with human post CM52 CETP. As well as Balb/c mice used for immunisation, a hybrid strain of mice (F_1 hybrids of CBA and Balb/c mice) were also immunised. The hybrid animals are more vigorous and often show a stronger antibody response. The mice immunised with post CM52 CETP showed the most inhibitory activity. These mice were boosted four times three weeks apart and tested each time. Tail bleeds were checked for activity both by inhibition of activity in a CETP assay and by their reaction against partially purified post CM52 CETP in an ELISA. The two best reactions in both the CETP assay and the ELISA were mice A_1 and A_5 (12% inhibition and 50% inhibition respectively at 1/20 dilution). The animals were boosted again but only one survived. The spleen from A_5 was fused with NS-1 cells. Again the fused cell colonies were slow growing. Dilutions from the colonies were screened both by the inhibition of CETP activity and by ELISA. It appeared that there were false positives in the CETP activity inhibition assay since positives in this assay did not always prove positive in the ELISA. At the last dilution three clones 24,16 and 20 were positive. Only clone 24 produced sufficient ascites to test and this proved negative. Purified (by ammonium sulphate precipitation and Sephadex G200 column) antibody from this ascites was also negative. It was decided to regrow clones from an earlier dilution stage which had proved positive in both the activity assay (60% inhibition) and the ELISA (0.15 $OD_{460\text{-}650nm}$). Clones D.HU.2.1E and D.HU.2.6H were regrown and injected into mice to produce antibodies in ascites. Both clones proved positive in a CETP inhibitory assay (Fig. 4.16). However in an ELISA only clone D.HU.2.6H reacted with human post CM52 CETP bound to the plate (Fig 4.17).

Since this ELISA used only a partially purified CETP preparation to coat the plate the clone may have been reacting to something other than CETP. To investigate further, a Western blot was carried out to see whether the clone would produce a signal at the correct molecular weight for the human CETP. This experiment proved inconclusive since although a band at approximately the correct molecular weight reacted with the ascites, many other bands reacted too, including a strong serum albumin reaction which partially obscured detection of a CETP reaction. Since ascites will contain mouse antibodies it is possible that the cross-reactivity may be due to reaction with other proteins. It is also possible that the clone was not a true clone and several antibodies were being produced by different cell lines. Limitations on time precluded further investigation of this promising clone. Further steps would be

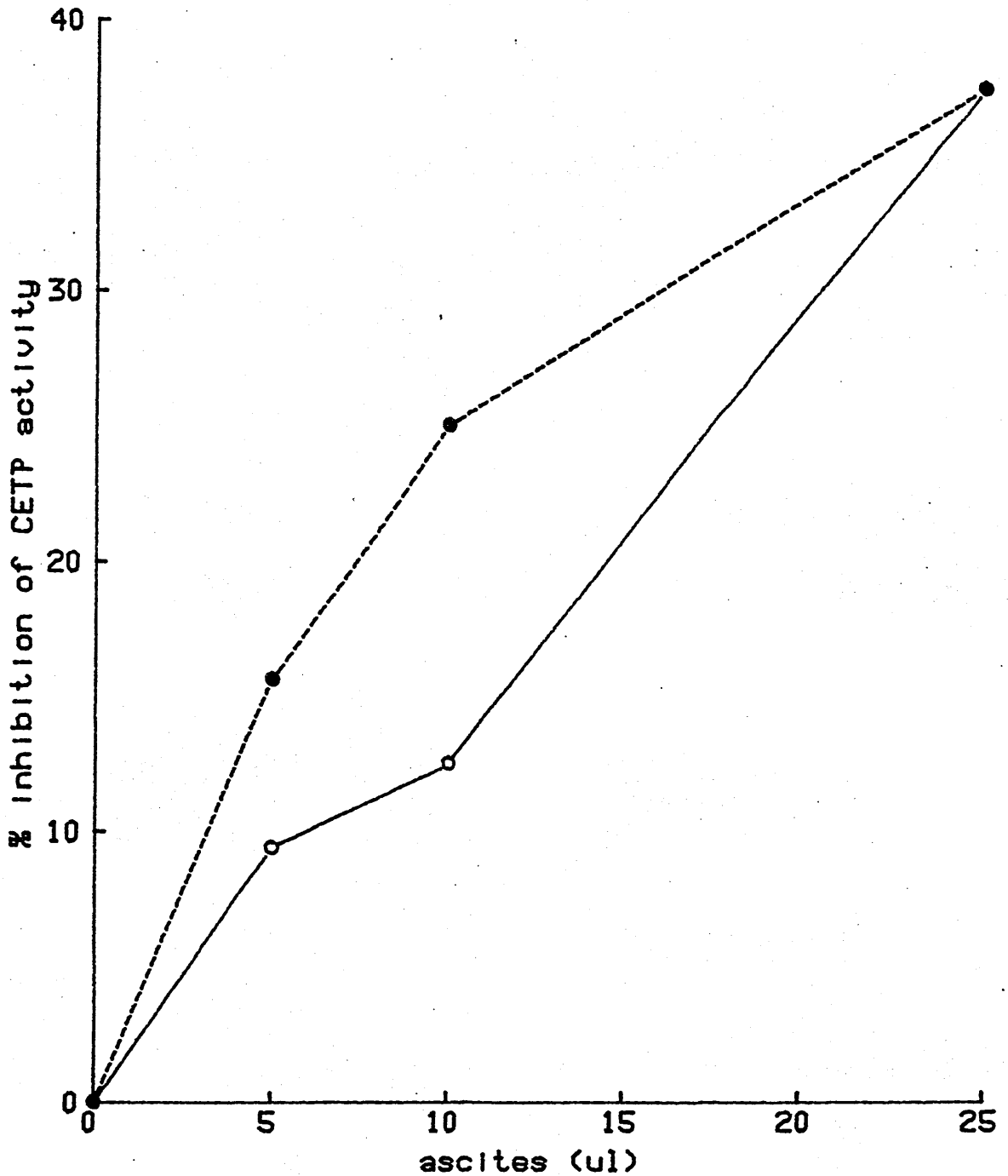


FIGURE 4.16 CETP ASSAY FOR THE INHIBITORY EFFECTS OF CLONES 2.1E AND 2.6H ANTI-HUMAN CETP SUPERNATANT. Mice were innoculated with cells from either the 2.1E clone (●) or the 2.6h clone (○). Ascites were tapped after three weeks. The ascites was added to a CETP assay containing a control amount of human LPDS.

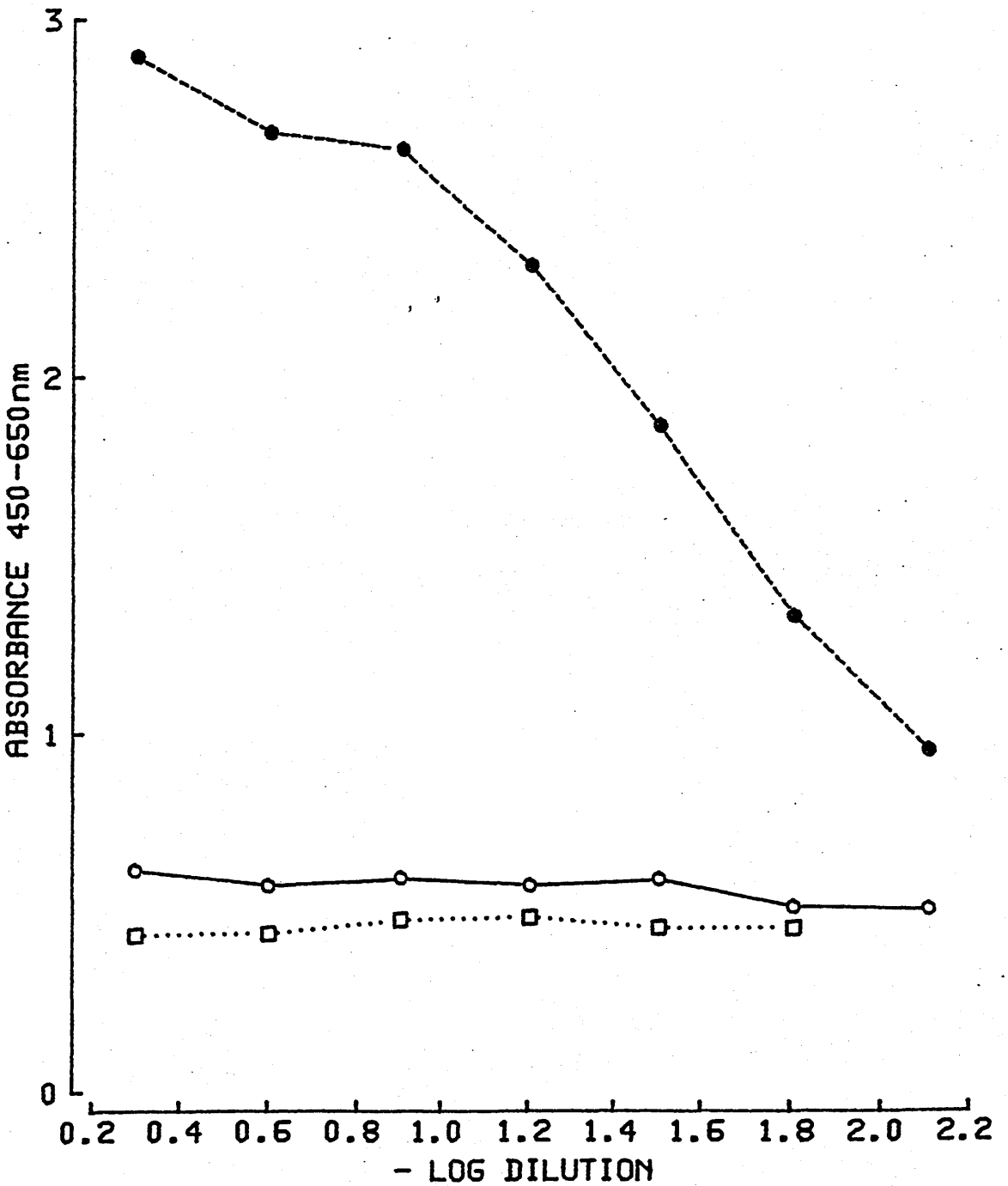


FIGURE 4.17 **ELISA OF MONOCLONAL ANTIBODIES TO HUMAN CETP.** Supernatants from the clones produced from the fusion of spleen cells from a mouse immunised with human post CM52 CETP with NS-1 cells were tested for their activity against human post CM52 in an ELISA. Plates were coated with 50μg/ml post CM52 CETP. The second antiserum was 1/1000 goat anti-mouse IgG conjugated to horse radish peroxidase. (○) clone 2.1E, (●) clone 2.6H, (□) non immune ascites.

to attempt to purify the antibody from the ascites and see whether a more specific reaction was obtained. If not then the clone D.HU.2.6H could be recloned and further ascites produced and tested.

CHAPTER V

STUDIES OF CETP ACTIVITY IN ANIMALS

1 Introduction

Hypercholesterolaemia is one of the risk factors associated with atherosclerosis. It has been noted that hypercholesterolaemia in rabbits induced either by diet or by a genetic defect is accompanied by an increase in plasma CETP activity. It is not known whether this is a cause and effect relationship or whether these parameters are independently controlled. Plasma CETP activity has been negatively correlated with HDL cholesterol levels in man but there is no strong correlation with total cholesterol. It is of interest to see whether manipulation of plasma total and HDL cholesterol levels affect CETP activity and whether CETP activity can be affected independently of plasma cholesterol. Various drugs can can alter plasma cholesterol levels as can feeding various diets. The effects of fat feeding, hereditary hyperlipidaemia, ACAT inhibitors, HMGCoA reductase inhibitors, probucol and other antioxidants were investigated in rabbit and marmoset models of hyperlipidaemia. Some studies were also carried out in humans.

2 Studies in the Rat

The rat is a species which expresses low levels of plasma CETP activity. This low expression of CETP is reflected in part by the lipoprotein metabolism of the rat. Most of the circulating cholesterol in the rat is carried by HDL and rat has a higher proportion of HDL than a human or a rabbit [Chapman, M.J. (1986)]. HDL in the rat has been demonstrated to deliver cholesterol to certain steroidogenic tissues [Gwynne, J.T. & Hess, B. (1980), Glass, C. *et al* (1983)]. This predominance of HDL might be expected from an animal which expresses low levels of CETP since CE will not be transferred out of the HDL. This raises questions as to how the rat carries out reverse cholesterol transport. It may be that HDL delivers cholesterol back to the liver [Quarfordt, S. *et al* (1980), Glass, C. *et al* (1983) and Arbeeny, C.M., Rifici, V.A. & Eder, H.A. (1987)] or possibly there is a transient expression of CETP activity to allow the cholesterol to be transported to lower density lipoproteins and taken back to the liver in that way. Rats show circadian rhythms [Parker, T.S. *et al* (1982)] in the expression of other enzymes of cholesterol metabolism e.g. HMGCoA reductase, so it may be possible that CETP may display such a rhythm too.

In order to test this hypothesis two groups of five male Wistar rats were kept in opposite light/dark cycles such that when blood was sampled one group of rats was six hours into the light cycle and one group was six hours into the dark cycle. Blood samples were taken from the tails of these ratsup to six weeks into the cycle. The CETP activities in the two groups of rats over the period of the experiment are shown in Fig. 5.1. The amount of CETP measured in LPDP prepared from the plasma sample was very low and hence there are very large errors on the measurements. The CETP levels of the rats measured in the mid-dark cycle was higher than that of the rats mid-light cycle up to four weeks but at six weeks was lower. The difference between mid light cycle rats and mid dark cycle rats was only significant at six weeks (Table 5.1) but was in the opposite direction from the previous two measurements. The differences detected were not particularly large when compared to the amount of CETP in other species blood (up to 100 times greater in humans) or that can be revealed by passage of rat plasma over a phenyl Sepharose column to remove inhibitor [Tollefson, J.H. *et al* (1988)]. Before passage over the column rat plasma CETP activity was 1% that of human. After passage over the column rat activity was 50 to 100% that of human CETP activity, which is a large increase. Since no difference in CETP activity could be detected between rats in the mid-light cycle and those in the mid-dark cycle except at six weeks, it appears that expression of CETP may be the same throughout the light/dark cycle and no circadian rythmn is present. It is possible that a large amount of CETP may be expressed at time points other than the middle of the light cycle and the middle of the dark cycle and these changes would be missed by the restriction to these two time points. This would mean that CETP would be peaking at a time different from the other enzymes and also that the peak of activity would be very sharp.

3 Studies in the Rabbit

5.3.1 Effects of Hypercholesterolaemia on Plasma CETP Activity

Rabbits have proved to be a useful model of atheroma as they can be made to produce lesions by cholesterol feeding or casein/sucrose feeding over several months. The plasma cholesterol levels which are achieved do tend to be extremely high. Watanabe rabbits are a strain that are genetically deficient in LDL receptors and also show very high plasma cholesterol and develop lesions. Both these rabbit models of hyperlipidaemia have been shown to express high levels of CETP activity [Son, Y-S. C. & Zilversmit, D.B. (1986)]. Froxfield heritable hyperlipidaemic (HH) rabbits (Southampton) are a breed which show a heritable hyperlipidaemia also due to an LDL receptor defect. Homozygotes show extremely high plasma cholesterol levels >10mM, develop lesions and are a good atherosclerotic model.

TIME AFTER INSTIGATION OF LIGHT/DARK CYCLE	CETP ACTIVITY MID LIGHT (%t/μg)	CETP ACTIVITY MID DARK (%t/μg)
1 WEEK	0.012 +/- 0.007	0.040 +/- 0.025
4 WEEKS	0.007 +/- 0.004	0.036 +/- 0.027
6 WEEKS	0.016 +/- 0.009	0.007 +/- 0.005*

* = P<0.05

TABLE 5.1 COMPARISON OF RAT PLASMA CETP LEVELS MID-DARK AND MID-LIGHT CYCLE. Plasma CETP levels were measured at 1, 4 and 6 weeks for each group of five rats after putting the rats into opposite light/dark cycles. For the group which were sampled in the mid-dark cycle, there was a significant drop (P<0.05) in CETP activity between week 1 and week 6. There was also a significant difference between the two groups at week 6 (P<0.05).

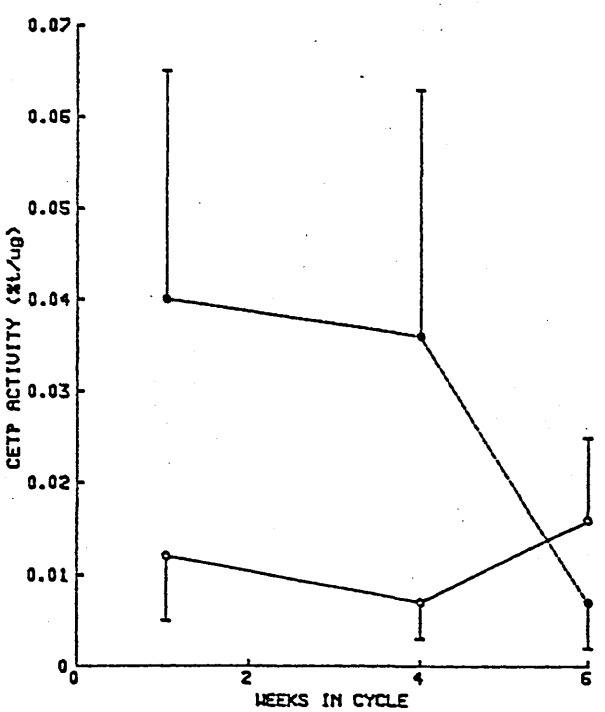


FIGURE 5.1 CETP ACTIVITY IN THE RAT DURING ADAPTATION TO LIGHT/DARK CYCLE. Two groups of five rats were kept in a 12 hour light/dark cycle such that at sampling one group was 6 hours into the light cycle (○) and one group was six hours into the dark cycle (●). Blood samples were taken from the tail vein at 1, 4 and 6 weeks. LPDP was prepared and CETP activity measured.

Plasma cholesterol levels and plasma CETP activities were compared in normal half lop rabbits (the normal strain bred with the HH rabbits) and Froxfield HH rabbits. Plasma cholesterol levels were significantly higher ($P<0.001$) in the Froxfield rabbits ($n=6$) than in the control half lops ($n=10$) at age 21 weeks (Table 5.2). Plasma CETP levels were also significantly higher ($P<0.05$) in these rabbits. Thus in this other example of genetically hyperlipidaemic rabbit, plasma CETP levels are demonstrably high.

To test whether fat feeding had the expected effect on plasma CETP levels, Dutch Belted rabbits on a high fat diet were compared to control rabbits. Plasma total cholesterol levels and plasma CETP levels were significantly higher in the fat fed rabbits than in the controls (Table 5.3). Levels in the fat fed rabbit were 70% higher than the control value. Thus these data confirm those already reported in the literature. Within the fat fed group plasma cholesterol was significantly correlated to CETP activity ($r=0.73$, $P<0.05$) but there was no correlation within the control group.

5.3.2 ACAT Inhibitor

Acyl CoA: cholesterol acyl transferase (ACAT) is a microsomal enzyme which esterifies cholesterol in cells thus allowing it to be stored as cholesteryl ester in the tissue. ACAT may be involved in the uptake of cholesterol from the gut. It has been suggested that ACAT inhibitors would be good antiatherosclerotic drugs because they could inhibit cholesterol uptake by the gut and/or prevent CE deposition in peripheral tissues. It was tested to see whether ACAT inhibitors would affect CETP e.g. by increasing the amount of free cholesterol in the cell which may be transported to the liver.

Rabbits were fed GR69918X, an ACAT inhibitor from Cyanamid made by Lancaster Synthesis (CL 277082), for 12 weeks at either 30 mg/kg/day ($n=10$) or 100 mg/kg/day ($n=10$) for 12 weeks. Control ($n=10$) animals were fed vehicle only. The animals were 21 weeks old at the end of the experiment. Total cholesterol, HDL cholesterol and plasma CETP activity were measured (Table 5.4). GR69918X had no effect on total plasma cholesterol but did significantly increase HDL cholesterol levels at 100 mg/kg/day. Animals treated with 30mg/kg/day GR69918X showed a non significant intermediate increase in HDL levels. CETP activity was not affected by treatment. Thus a change in HDL cholesterol was not accompanied by a change in CETP activity. It has been proposed that there is an inverse correlation between plasma HDL levels and CETP activity [Groener, J.E.M. *et al* (1984), Tollefson, J.H. *et al* (1988)] thus we might have expected to see a fall in CETP activity concomitant with the rise in HDL, but this was not the case. However the HDL levels measured here are very low and have probably been underestimated since the technique used was suitable for human samples but had not been validated for hyperlipidaemic rabbits. No ACAT inhibitor has yet been demonstrated to decrease plasma cholesterol but this may be due to problems with bioavailability.

	TOTAL CHOLESTEROL (mM)	CETP ACTIVITY (%t/μg)
NORMAL RABBIT (HALF LOP)	2.10 +/- 1.78	0.572 +/- 0.101
HYPERLIPIDAEMIC RABBIT (HHL)	13.77 +/- 4.17*	0.839 +/- 0.203**

* P= 0.001
** P= 0.05

TABLE 5.2 **PLASMA TOTAL CHOLESTEROL LEVELS AND CETP ACTIVITY IN NORMAL AND GENETICALLY HYPERLIPIDAEMIC RABBITS.** Plasma total cholesterol levels and CETP activities were measured in 10 control half lop rabbits and 6 Froxfield HH rabbits at 21 weeks of age.

	TOTAL CHOLESTEROL (mM)	CETP ACTIVITY (%t/μg)
CONTROL DIET	0.87 +/- 0.34	0.185 +/- 0.022
HIGH FAT DIET	5.49 +/- 3.78*	0.316 +/- 0.077**

* = P<0.05
** = P<0.001

TABLE 5.3 **TOTAL CHOLESTEROL LEVELS AND CETP ACTIVITY IN RABBITS FED CONTROL AND HIGH FAT DIETS.** Dutch Belted rabbits were fed either control (FD1) (n=4) or low cholesterol, high fat diet (n=8) for 11 weeks. Blood samples were taken from the marginal ear vein and serum prepared. Total cholesterol was measured and CETP activity measured in the LPDS.

TREATMENT	TOTAL CHOLESTEROL (mM)	HDL CHOLESTEROL (mM)	CETP ACTIVITY (%t/μg)
CONTROL (n=10)	19.85 +/- 4.70	0.29 +/- 0.14	1.08 +/- 0.30
GR69918X 100mg/kg/day (n=10)	22.58 +/- 5.19	1.03 +/- 0.50*	1.17 +/- 0.27_{NS}

* - P<0.001
NS - not significant

TABLE 5.4 **EFFECT OF AN ACAT INHIBITOR ON TOTAL CHOLESTEROL LEVELS AND CETP ACTIVITY IN FROXFIELD RABBITS.** Total cholesterol, HDL cholesterol and CETP activity were measured in serum samples from control Froxfield rabbits or Froxfield rabbits fed either 30mg/kg/day or 100mg/kg/day GR69918X, an ACAT inhibitor (Cyanamid). Each group contained 10 rabbits. The data for the 30mg/kg/day group is not shown.

TREATMENT	TOTAL CHOLESTEROL (mM)	CETP ACTIVITY (%t/μg)
CONTROL (n=12)	17.44 +/- 5.73	1.14 +/- 0.36
PROBUCOL (n=12)	13.45 +/- 3.76_{NS}	1.07 +/- 0.38_{NS}

NS = not significant

TABLE 5.5 **THE EFFECT OF PROBUCOL ON TOTAL CHOLESTEROL AND CETP ACTIVITY IN FROXFIELD RABBITS.** One group of 12 rabbits was fed normal FD1 diet and one group of 12 rabbits was fed 1% probucol incorporated into FD1 diet for 12 weeks. Blood samples were taken from the marginal ear vein. Serum total cholesterol was measured and CETP activity in LPDS was assayed.

5.3.3 Salbutamol

Salbutamol is a β_2 adrenergic agonist. It has been reported that cAMP stimulation of neutral cholesteryl ester hydrolase (NCEH) can lead to a decrease in cellular CE content in aortic smooth muscle cells [Hajjar, D.P. & Weksler, B.B. (1983)]. Since salbutamol works via a cAMP dependent mechanism it was tested to see whether this drug would have any effect on cholesterol metabolism in rabbits. If less CE are stored in the cell then more free cholesterol is available for removal from peripheral cells which may lead to a rise in CETP activity. A study was performed where total cholesterol and CETP activity was measured in 12 control HH rabbits (fed FD1 diet) and 12 HH rabbits fed FD1 diet containing 0.03% salbutamol for 12 weeks. At the end of the experiment there was no significant difference in total cholesterol and CETP activity between treated and control groups (data not shown).

5.3.4 Probucol

Probucol (Merrell Dow) is a drug that has been shown to have good lowering effects on plasma total cholesterol but is not widely used in the clinic because it also results in a disproportionate decrease in HDL levels [Beyen, A.C. (1986), Illingworth, D.R. (1987)]. However, recently it has been shown that probucol can prevent lesion growth in Watanabe rabbits [Kita, T. *et al* (1987)]. The mode of action of this drug remains obscure but it is known to be an antioxidant [Barnhart, R.L., Busch, S.J. & Jackson, R.L. (1989)]. It has been suggested that the drug may protect circulating LDL from oxidative modification and so may prevent its uptake by the scavenger receptor into macrophages leading to foam cell development and lesion formation [Naruszewicz, M. *et al* (1984), Parthasarathy, S. *et al* (1986), Carew, T.E., Schwenke, D.C. & Steinberg, D. (1987)]. Apart from these effects it has also been suggested that probucol may increase impaired plasma CETP activity [Matsuzawa, Y. *et al* (1988), Sirtori, C.R. *et al* (1988), Franceschini, G. *et al* (1989)] possibly resulting in increased flow of cholesterol out of peripheral tissues.

A group of twelve Froxfield HH rabbits were treated with 1% probucol incorporated into their diet and effects on plasma total cholesterol and CETP activity were measured at the end of 12 weeks and compared with a group of twelve control rabbits fed normal diet. Probucol did not significantly decrease plasma total cholesterol levels nor did it significantly decrease plasma CETP activity (Table 5.5). Since there was no effect on total cholesterol then it might not be expected that CETP would be affected as the rabbits were obviously not absorbing enough probucol to have a cholesterol lowering effect. However it is shown that probucol does not independently affect CETP in these rabbits. Plasma probucol levels were 145 +/- 18.2μM so probucol was being absorbed by these rabbits. The probucol treated rabbits showed a reduced amount of lesion development compared with controls (work carried out by Jo

Motteram, Glaxo Group Research Ltd, Greenford), thus a lesion effect of the drug was apparent. This evidence does not appear to support a mechanism for CETP in reduction of lesion progression.

5.3.5 Other Antioxidants

The effect of antioxidants on plasma CETP activity was studied again using another novel antioxidant, GR44966X. Probucol was used in the same experiment as a reference. Froxfield HH rabbits were fed for five days with control diet or diet containing 1% probucol, 0.1% probucol or 0.1% GR44966X. GR44966X was fed in two forms; a) unused diet from a previous experiment, which was four weeks old, for five days and b) freshly prepared diet containing GR44966X for 3 days. Plasma CETP activity was not significantly affected by probucol (Table 5.6). However in the case of GR44966X, CETP activity was halved. This decrease was significant in the case of treatment (b), but not in the case of treatment (a). Group (b) showed a large reduction in CETP activity even though these animals were only on the diet for three days. Here, it seems, is an agent which can reduce CETP activity. Unfortunately in this particular experiment plasma cholesterol levels were not measured. It is not known whether GR44966X has a direct effect on CETP activity when added directly to an assay.

Probucol has been proposed to increase CETP activity. Therefore it is interesting to see that an agent which also an antioxidant appears to decrease plasma CETP activity. Since the numbers in the animal groups here are so small it is obviously desirable to repeat this experiment with larger animal groups as this may just be a chance observation.

4 Studies in the Marmoset

Marmosets are primates and exhibit cholesterol levels similar to those found in man (about 4.5mM) but it appears that they have a greater proportion of cholesterol in their HDL fraction. They are therefore a useful model for atherosclerosis since the cholesterol levels are not extreme. HMGCoA reductase inhibitors inhibit the biosynthesis of cholesterol and have profound effects on plasma cholesterol levels. In the marmoset model where marmosets were treated with GR92549, an HMGCoA reductase inhibitor, reductions of 37% (at 10mg/kg/day) and 47% (at 30mg/kg/day) in plasma total cholesterol were seen after seven days treatment. These reductions were significant (Table 5.7). Control animals also showed a significant but smaller decrease in plasma total cholesterol (20%). This may be due to stress or unspecified effects due to dosing. CETP activity was measured in each of the treatment groups at day 0 and day 7. The only significant change in CETP activity occurred in the control group where activity was decreased by 37%. Cholesterol lowering by the HMGCoA reductase inhibitor did not bring about lowering of plasma CETP activity. The decrease in the activity in the control may or may not be related to the total cholesterol lowering in the control. If it is due to this cholesterol lowering then the mechanism for this cholesterol lowering effect may different

TREATMENT	CETP ACTIVITY (%t/μg)	NUMBER IN GROUP	P VALUE
SDS DIET (5 days X 100g)	1.29 +/- 0.20	3	
0.1% PROBUCOL (5 days X 100g)	1.47 +/- 0.22	3	0.39
1% PROBUCOL (5 days X 200g)	1.17 +/- 0.47	3	0.72
0.1% GR44966X(a) (5 days X 100g)	0.66 +/- 0.33	3	0.06
0.1% GR44966X(b) (3 days X 100g)	0.46 +/- 0.23	2	0.05

TABLE 5.6 **THE EFFECT OF AN ANTIOXIDANT, GR44966X, ON CETP ACTIVITY IN FROXFIELD RABBITS.** Froxfield rabbits were divided into 5 groups and fed various diets as follows:- one group of 3 was fed normal SDS diet for 5 days at 100g/day, one group of 3 was fed diet with 0.1% probucol incorporated for 5 days at 100g/day, one group of 3 was fed diet with 1% probucol incorporated for 5 days at 200g/day, one group of 3 was fed diet with 0.1% GR44966X incorporated (diet was four weeks old) for 5 days at 100g/day and finally one group of 2 rabbits was fed 0.1% GR44966X for 3 days at 100g/day (diet made fresh at start of experiment). Blood samples were taken from the marginal ear vein. CETP activity was measured in LPDS prepared from the sample.

TREATMENT	TOTAL CHOLESTEROL (mM)		CETP ACTIVITY (%t/μg)	
	DAY 0	DAY7	DAY0	DAY7
CONTROL (n=6)	4.79 +/- 0.36	3.84* +/- 0.20	0.35 +/- 0.09	0.22+ +/- 0.05
GR92549 10 mg/kg/day (n=6)	4.39 +/- 0.09	2.77** +/- 0.13	0.19 +/- 0.08	0.27 +/- 0.11
GR92549 30mg/kg/day (n=6)	5.14 +/- 0.05	2.71*** +/- 0.36	0.31 +/- 0.12	0.29 +/- 0.05

* P<0.005 ** P<0.001 *** P<0.05
\+ P<0.05

TABLE 5.7 **THE EFFECT OF AN HMGCoA REDUCTASE INHIBITOR, GR92549, ON TOTAL CHOLESTEROL LEVELS AND CETP ACTIVITY IN MARMOSETS.** Marmosets were divided into three groups of 6. One group was a control group, a second group was dosed at 10mg/kg/day GR92549 for 7 days and a third group was dosed at 30mg/kg/day GR92549 for 7 days. Blood samples were taken from the tail vein and assayed for total cholesterol and CETP activity was measured in the LPDS.

than that employed by HMGCoA reductase inhibitors. These marmosets demonstrated plasma cholesterol lowering which was not accompanied by a reduction in plasma CETP activity.

5 Studies in Humans

Two drug studies were carried out in humans. The first study was to investigate the effect of probucol on plasma CETP activities in normal volunteers. The second was to look at the plasma CETP activities in hypercholesterolaemic patients being treated in the clinic with an HMGCoA reductase inhibitor, Simvastatin.

5.5.1 Probucol

It has been reported that probucol treatment can lead to an increase in plasma CETP activity. This was not found to be the case in the rabbit study. To see if there might be any effect in humans, a pilot study looking at the effect of two weeks treatment at a dose of 500mg probucol twice daily in two volunteers. In this pilot study a fall in total cholesterol from 5.76 +/- 0.47mM to 4.83 +/- 0.02 mM was seen (Table 5.8). This was accompanied by a fall in HDL cholesterol from 1.32 +/- 0.12mM to 1.13 +/- 0.13mM. Total TG did not seem to be affected. CETP activity measured in the LPDS from these two patients was increased 1.8 fold in treated samples. Control activities were 0.094 +/- 0.002 %t/μg and after treatment were 0.173 +/- 0.001 %t/μg. An investigation was then carried out to see whether there were changes in the ability of the volunteers' lipoproteins to act as donors or acceptors. Assays using ^{3}H-HDL$_3$ or LDL/VLDL from each donor in a CETP assay with a standard amount of LPDS showed that there were increased transfer rates with the same amount of LPDS using lipoproteins from the post probucol samples as opposed to the control samples. Thus the increase in plasma CETP activity may be at least partly due to increased ability of the lipoproteins to act as substrates. LCAT activities were not greatly affected by Probucol treatment. Probucol when added to an *in vitro* assay in concentrations up to 500μM showed no significant direct effect on CETP activity, though there were difficulties in getting the drug dissolved in a solvent which would not interfere with the assay.

The results of this pilot study were interesting but the sample size was not large enough to test whether the effect was statistically significant. Therefore the study was repeated using six healthy male volunteers. Again the schedule was taking 500mg probucol twice daily for two weeks. A 200ml blood sample was taken at day 0 and day 14.

The results of the larger probucol study are shown in Fig. 5.9. Plasma total cholesterol was significantly reduced from 5.65 +/- 1.08 mM to 4.70 +/- 1.01 mM ($P<0.005$). HDL cholesterol was also significantly reduced from 1.22 +/- 0.14 mM to 0.86 +/- 0.14mM ($P<0.001$). Triglycerides were unaffected. CETP activities were measured in LPDS and there was found to be no significant difference between control 0.259 +/- 0.051 %t/μg and post Probucol samples 0.301 +/- 0.078 %t/μg.

	TIME = 0	TIME = 2 WEEKS
TOTAL CHOLESTEROL (mM)	5.76 +/- 0.47	4.83 +/- 0.02
HDL CHOLESTEROL (mM)	1.32 +/- 0.12	1.13 +/- 0.13
TRIGLYCERIDES (mM)	0.66 +/- 0.09	0.58 +/- 0.03
CETP ACTIVITY using standard LP substrates (%t/μg)	0.094 +/- 0.002	0.173 +/- 0.001
CETP ACTIVITY using standard LPDS LDL/VLDL (%t/μg)	0.065 +/- 0.004	0.089 +/- 0.003
CETP ACTIVITY using standard LPDS ^{3}H-HDL_3 (%t/μg)	0.104 +/- 0.003	0.157 +/- 0.045
LCAT ACTIVITY (pmol/hour/μg)	0.628 +/- 0.106	0.602 +/- 0.151

TABLE 5.8 **MEASUREMENTS OF PLASMA LIPIDS AND ENZYME ACTIVITIES IN HUMANS TREATED WITH PROBUCOL.** Two human male volunteers were treated with 500mg probucol twice daily for 2 weeks. Blood samples were taken at the start and finish of the experiment. Plasma total cholesterol, HDL cholesterol and triglycerides were measured. CETP activity and LCAT activity were measured in the LPDP using standard substrates. LDL/VLDL and HDL_3 from the volunteers were also tested for their ability to act as substrates in a CETP assay using a standard amount of LPDS.

	TIME = 0	TIME = 2 WEEKS
TOTAL CHOLESTEROL (mM)	5.65 +/- 1.08	4.70 +/- 1.01*
HDL CHOLESTEROL (mM)	1.22 +/- 0.14	0.86 +/- 0.14**
TRIGLYCERIDE (mM)	0.95 +/- 0.36	0.81 +/- $0/34_{NS}$
CETP ACTIVITY using standard LP substrates (%t/μg)	0.259 +/- 0/051	0.301 +/- 0.078_{NS}
LCAT ACTIVITY (pmol/hour/μg)	0.551 +/- 0.223	0.519 +/- 0.048_{NS}

* = P<0.005
** = P<0.001
NS = not significant

TABLE 5.9 **MEASUREMENTS OF PLASMA LIPIDS AND ENZYME ACTIVITIES IN HUMAN VOLUNTEERS TREATED WITH PROBUCOL - LARGE STUDY.** Six human male volunteers were treated with 500mg probucol twice daily for two weeks. Blood samples were taken at the start and the finish of the experiment. Plasma total cholesterol, HDL cholesterol and triglycerides were measured. CETP activity and LCAT activity were measured in the LPDP using standard substrates.

Using a standard LPDS and using LDL/VLDL or ^{3}H-HDL_3 from each volunteer it was seen that there was no difference in the ability of LDL/VLDL or HDL_3 to act as substrates. LCAT activities were also not affected by probucol.

Thus the findings of the pilot study were not supported by the findings of the larger study. The effects seen were not statistically significant. The plasma CETP activities in the pilot study even after probucol treatment were at the low end of the range of values obtained for the six volunteers. Possibly for some reason CETP levels were depressed in the two volunteers prior to treatment and probucol returned them to a near normal value or they may have been returning to 'normal' anyway.

In anticipation that the pilot study results where plasma CETP levels were increased in the post probucol samples would be repeated in the second study, samples of plasma from the second study were sent to John Albers (Northwest Lipid Research Clinic, Seattle) who had offered to test whether there was any change in plasma CETP inhibitor levels after treatment. His analysis also ascertained that there was no significant difference in activity between control and treated samples (Fig. 5.2). Further analysis on these samples was carried out. Partially purified CETP was added to each plasma sample and then the samples were assayed for total CETP activity. Significantly more activity was found in the post probucol samples and they concluded that the post probucol sample may contain less inhibitor than the control sample.

5.5.2 HMG CoA Reductase Inhibitor

The studies on Simvastatin were carried out in conjunction with Dr Luciano Comminacini at the Verona Institute. Plasma samples were sent from patients who had received Simvastatin according to the dosing regime shown below:-

T0 - before starting therapy
T45 - 45 days at 10mg per day
T90 - 45 days at 10mg/day and 45 days at 20 mg/day
T135 - 45 days at 10mg/day, 45 days at 20mg/day and 45 days at 30mg/day
T180 - 45 days at 10mg/day, 45 days at 20mg/day, 45 days at 30 mg/day and 45 days at 40mg/day.

A group of eight patients was followed on this therapy. This group comprised four males and four females. All had Type IIa hyperlipidaemia. The plasma cholesterol levels and plasma CETP activities of this group are described in Fig. 5.10. At T0 neither total cholesterol nor HDL cholesterol was significantly correlated to CETP activity.

Fig. 5.3 A and B follows each patient through the course of their treatment. The patients seem to fall into two groups. The first, Fig. 5.3A, contains patients whose measured parameters behave in a similar way to each other throughout the experiment. Plasma total cholesterol levels fall, plasma HDL cholesterol levels rise and plasma CETP activities fall reflecting the fall in total cholesterol. The second group contains two patients; both show a fall in HDL cholesterol and one shows an

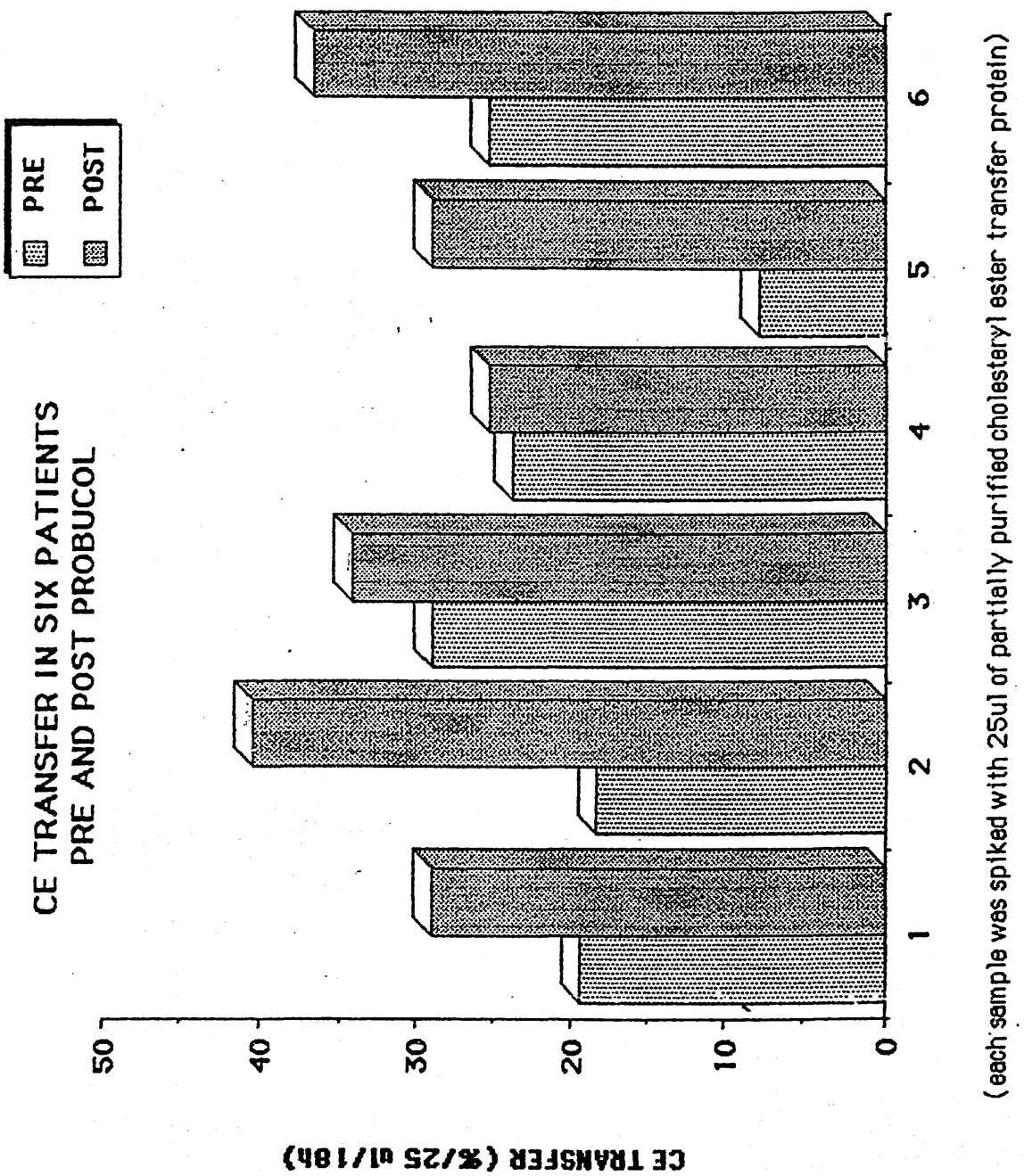


FIGURE 5.2 CHOLESTERYL ESTER TRANSFER IN SIX VOLUNTEERS PRE- AND POST- PROBUCOL TREATMENT. This experiment was carried out by Dr J. Albers' laboratory, Northwest Lipid Research Clinic, Seattle. Partially purified CETP (25µl) was added to 2ml of each pre and post probucol sample. The samples were then assayed for total CETP activity.

	CHOLESTEROL (mM)	CETP ACTIVITY (%t/μg)
MEAN	10.4	0.273
MEDIAN	9.9	0.223
S.D.	1.9	0.116
MIN	8.6	0.189
MAX	14.1	0.508

TABLE 5.10 DESCRIPTION OF EIGHT VERONA HYPERLIPIDAEMIC PATIENTS.

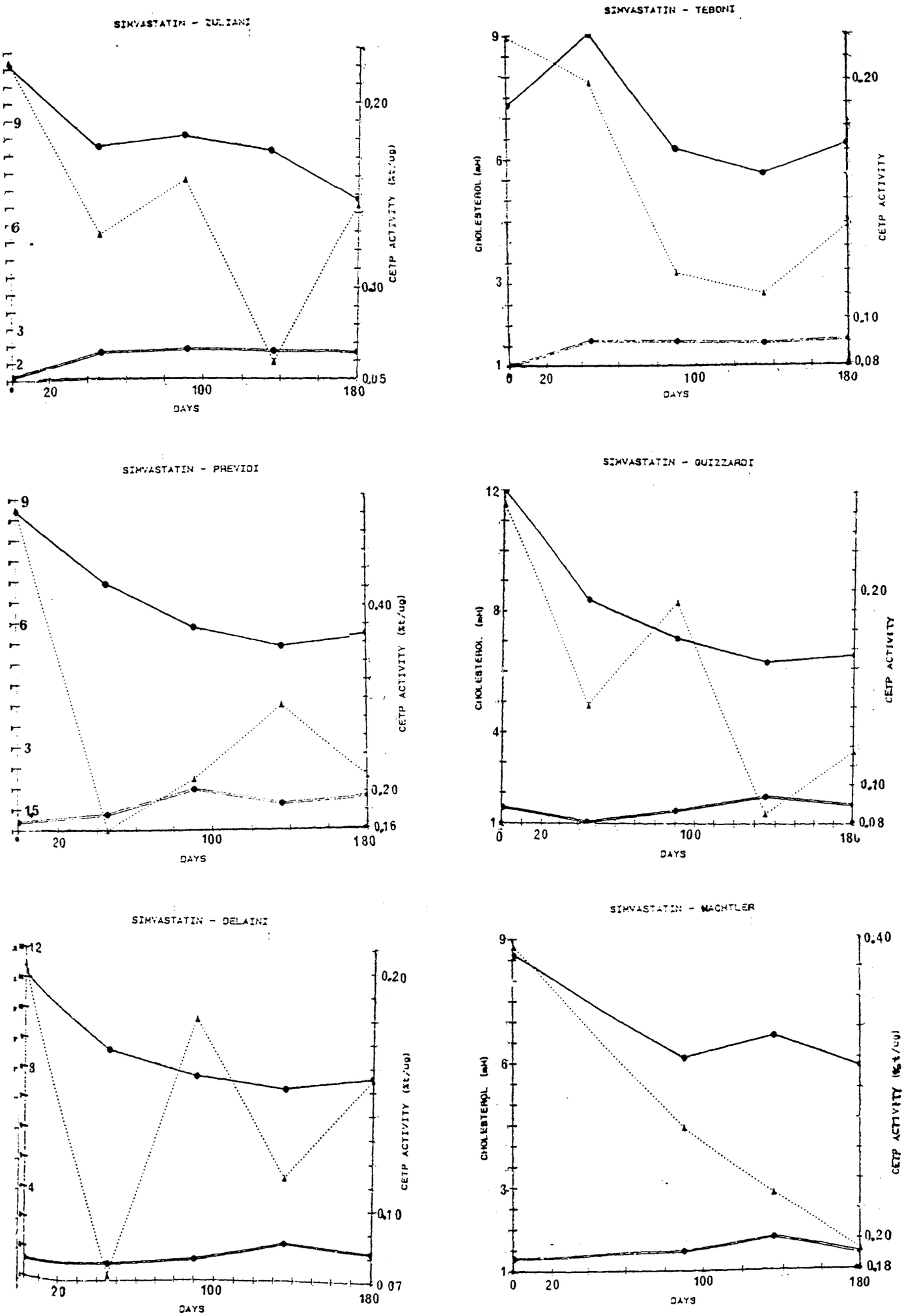
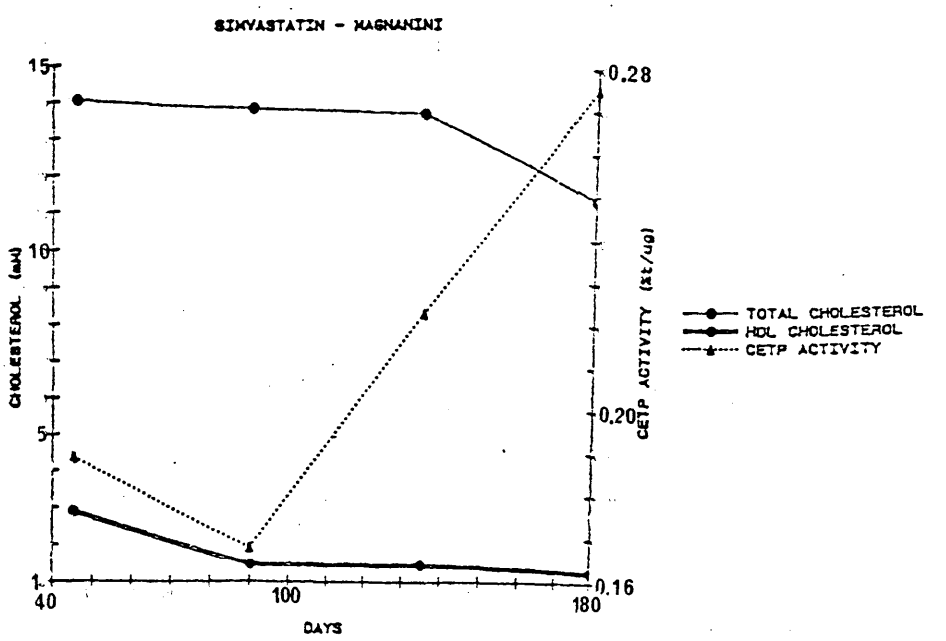

SIMVASTATIN - ZULIANI
SIMVASTATIN - TEBONI
SIMVASTATIN - PREVIDI
SIMVASTATIN - GUIZZARDI
SIMVASTATIN - DELAINI
SIMVASTATIN - MACHTLER
CHOLESTEROL (mM)
CETP ACTIVITY (%t/ug)
DAYS
TOTAL CHOLESTEROL
HDL CHOLESTEROL
CETP ACTIVITY

Fig 5.3A

Fig 5.3B



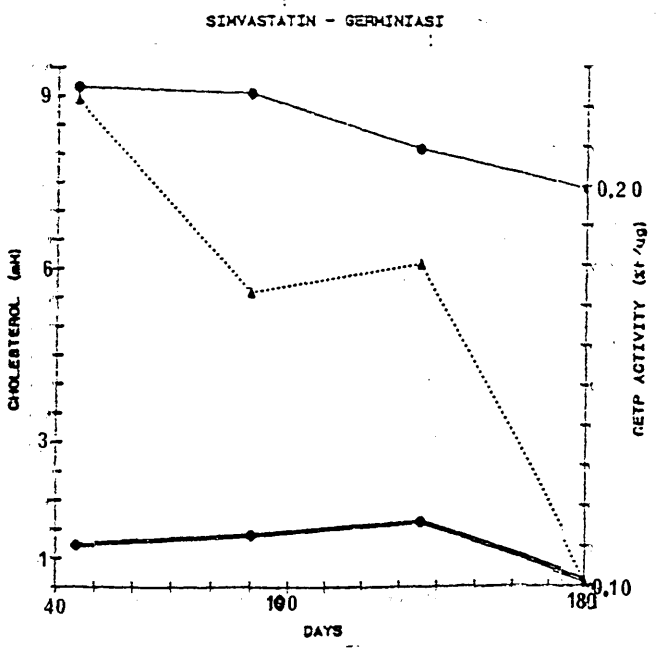


FIGURE 5.3 **TIME COURSE OF TOTAL CHOLESTEROL, HDL CHOLESTEROL AND CETP ACTIVITY FOR EIGHT PATIENTS TREATED WITH SIMVASTATIN.** Patients were treated with simvastatin according to the regime described in the text. Total cholesterol, HDL cholesterol and CETP activity were measured at 45 day intervals. The time course of the changes in these parameters is plotted. A) Six patients whose measured parameters vary in a similar way to each other throughout the experiment. B) Two patients who showed a different pattern from the rest.

increase in CETP activity. Looking over the information available for the patients the only thing that could be found that was remarkable about these two individuals was that one, Germiniasi, had the highest TG of the group, and the other, Magnanini, had the lowest TG of the group and a very high plasma cholesterol level (14mM).

Plasma total cholesterol, HDL cholesterol and CETP activity were compared at T=0 and T=180 days (Table 5.11). There was a significant ($P<0.001$) 32% decrease in total plasma cholesterol. This would be expected as simvastatin is a hypocholesterolaemic agent. HDL cholesterol was increased by 11% but this was not significant. HDL may play a protective role and so a fall in total cholesterol with a concomitant rise in HDL cholesterol will lead to a favourable LDL:HDL ratio. Plasma CETP activity was significantly reduced ($P<0.01$) by 41%. A significant fall in CETP activity was seen to accompany the fall in plasma total cholesterol levels in this experiment with humans whereas a similar experiment with marmosets, although showing an effect on total cholesterol demonstrated no effect on plasma CETP levels. There are two main differences between the studies. Firstly the time scale is quite different. The marmoset experiment was only over seven days whereas the human experiment was over 180 days. It is possible that there is a lag in any appearance of effect on CETP levels and so a change was not picked up in marmoset experiments. Secondly the group of humans which were being treated were very hypercholesterolaemic (mean total cholesterol level = 10.4mM) whereas the marmoset mean cholesterol was 4.7mM. Thus a decreasing effect on plasma CETP activity may only be apparent if activity is raised in the first place as in hypercholesterolaemia. Neither total cholesterol nor HDL cholesterol were significantly correlated to CETP in this population.

	TIME = 0	TIME = 180	% CHANGE
TOTAL CHOLESTEROL (mM)	10.4 +/- 1.9	7.1 +/- 1.9	-32%*
HDL CHOLESTEROL (mM)	1.6 +/- 0.6	1.8 +/- 0.9	$+11\%_{NS}$
CETP ACTIVITY (%t/μg)	0.273 +/- 0.116	0.160 +/- 0.065	-41%**

* = P<0.001

** = P<0.05

NS = not significant

TABLE 5.11 **THE EFFECT OF SIMVASTATIN ON TOTAL CHOLESTEROL, HDL CHOLESTEROL AND CETP ACTIVITY IN EIGHT HYPERLIPIDAEMIC PATIENTS.** Patients were treated with simvastatin according to the regime described in the text for up to 180 days. Total cholesterol, HDL cholesterol and CETP activity were measured in the first and last samples.

CHAPTER VI

REGULATION OF CETP SECRETION IN CELLS

1 Introduction

Cells have been demonstrated to secrete CETP in culture. Cell types studied include J774, a mouse macrophage-like cell line [Tollefson, J.H. *et al* (1985)], HepG2, a human hepatoma [Faust, R.A. & Albers, J.J. (1987)], CaCo-2, an enterocyte cell line [Faust, R.A. & Albers, J.J. (1988)] and human monocyte derived macrophages [Tollefson, J.H. *et al* (1985)]. It is possible that these cells represent the cells responsible for CETP production *in vivo*. Obviously the mass of CETP protein is a contributing factor to the overall expression of CETP activity. Being able to understand what controls the secretion of this protein may help provide insight into the significance of CETP. The variety of cell types which express CETP secretion is in itself confusing. Which are the most relevant cell types to study? Liver cells, intestinal cells and macrophage cells all secrete the protein. Intestinal cells may be important if CETP serves some post prandial function: plasma CETP activity is increased during alimentary lipaemia [Tall, A. *et al* (1986)] and free fatty acids have been shown to increase CETP secretion by CaCo-2 cells. The liver is central to cholesterol metabolism and HepG2 cells have been shown to secrete CETP. Also CETP has been detected in liver perfusates, possibly due either to hepatocytes or Kupffer cells. The liver may be able to secrete CETP in response to either hormones or in response to changes in plasma cholesterol levels. If CETP is involved in reverse cholesterol transport it would be important to regulate the amount of enzyme expressed so that cholesterol balance between the liver and the periphery can be maintained. Upsets in the balance may lead to atherosclerosis. Macrophages have been shown to be involved in lesion development and can form lipid laden foam cells within the lesion. CETP may be secreted by these cells in order to improve the flow of cholesterol into or out of cells. In this way CETP may be having localised effects.

There are a variety of hypothetical roles for CETP with the protein having either systemic or local actions. Studying the response of the cells to various effectors may highlight the importance of a particular mechanism. Culture systems can be very artificial and it is important to corroborate any findings by looking at the effect of any relevant effectors *in vivo*.

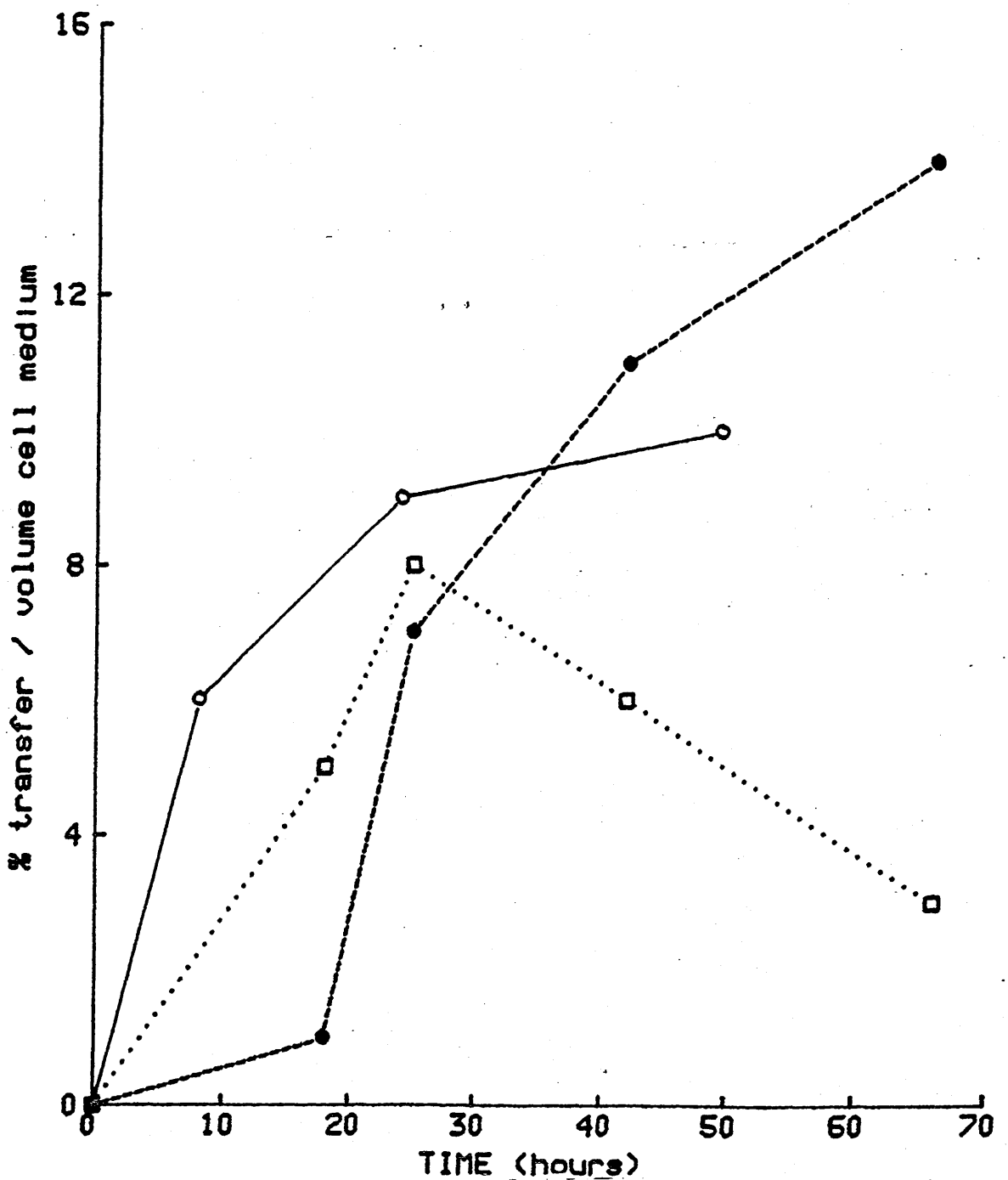


FIGURE 6.1 TIME COURSE OF CETP SECRETION IN HUMAN MONOCYTE DERIVED MACROPHAGES, J774 CELLS AND HEPG2 CELLS. Human monocyte derived macrophages were isolated as described in the methods and plated into 6 well plates. They were cultured for 7 days and then washed in serum-free medium and then incubated in serum-free medium for 8, 24 and 48 hours at 37°C. HepG2 cells were plated into 6 well plates and cultured as described in the methods. When they had reached 90% confluence they were washed with serum-free medium. J774 cells were washed with serum-free medium and then plated into 6 well plates at a density of $1x10^6$ cells/ml/well. The HepG2 cells and J774 cells were incubated in serum-free medium for 0, 18, 25, 42 and 66 hours. At each time point conditioned medium was collected and frozen at -70°C until they were assayed for CETP activity. Macrophages (○), J774 (●), HepG2 (□).

TREATMENT	TRANSFER SECRETED INTO MEDIUM (%t/40µl)
CONTROL	9.7 +/- 3.8
CON A (10µg/ml)	7
TPA (10µg/ml)	16
EtOH (10µl)	9
LPS (10µg/ml)	3
TGFβ (10ng/ml)	7
LDL (200µg/ml)	11
PROBUCOL LDL (200µg/ml)	3
HDL (200µg/ml)	11
PROBUCOL HDL (200µg/ml)	6
AcLDL (200µg/ml)	4
βVLDL (200µg/ml)	3

TABLE 6.1 **CETP SECRETION BY MACROPHAGES IN THE PRESENCE OF VARIOUS EFFECTORS.** Human monocyte derived macrophages were isolated and cultured for 8 days. The medium was then removed, the cells washed with serum free medium and the medium replaced with 1ml serum-free medium containing various agents as listed in the table. Cells were incubated in this medium for 24 hours at 37°C. The medium was then collected, frozen at -20°C and assayed for CETP activity.

which were more likely to have specific effects. PMA (Fig. 6.2A) at a lower dose, had no significant effect on CETP secretion. LPS (Fig 6.2B) also at a more physiological dose of 500ng/ml had little effect on CETP secretion though in these cells very little CETP was secreted anyway. The effect of IL-1, an immunoregulator and local hormone, on CETP secretion by macrophages was also studied. Firstly IL-1 was tested at one concentration; 10ng/ml over 48 hours. It appeared that IL-1 may have had some stimulatory effect at 48 hours though again secretion in the control cells was low. When a 10 fold higher concentration of IL-1 was tested over 24 hours no difference in CETP secretion was found between the control and either 10 or 100ng/ml IL-1 (Fig. 6.3).

Problems were encountered in the work with macrophages in that cells prepared from different donors would behave very differently from each other. Macrophages were obtained by harvesting blood circulating monocytes and differentiating them into macrophages by allowing them to adhere to a plastic culture plate coated with poly-D-lysine. Yields of cells found sticking to the plate varied largely between preparations. Also the amount of CETP secreted by cells from different donors varied a great deal. Since cells required autologous serum it was not feasible to pool blood from different donors to try and even out discrepancies. Thus the macrophages were not a very useful line with which to continue the work since their properties were so inconsistent.

J774 cells are a mouse macrophage-like cell line. They secrete CETP and the effects of PMA and various agents which interfere with cholesterol metabolism were studied in this cell line. PMA had a slight stimulatory effect on CETP secretion at 48 hours (Fig 6.4) but again in this experiment cell secretion was barely measurable. A collection of effectors which might affect the cholesterol balance of the cell were tested to see whether they had any effect on CETP secretion. These effectors were LDL, AcLDL and two drugs; an HMGCoA reductase inhibitor, mevinolin (sodium salt), and an ACAT inhibitor, 58-035 (Sandoz). J774 cells can accumulate cholesterol in response to exposure to AcLDL. They take up this modified LDL in an uncontrolled manner and store the cholesterol as CE in lipid droplets thus forming foam cells. LDL is taken up by a receptor which is tightly controlled resulting in down-regulation of the receptor and of HMGCoA reductase, and up-regulation of the esterifying enzyme ACAT. Fig. 6.5 shows the effects of these treatments on CETP secretion by J774 cells. There was no significant effect with any of these agents at 40 hours. However it appeared that at 15 hours there was an increase in CETP secretion showing a peak with AcLDL, mevinolin and 58-035. It is difficult to explain how these treatments had the same effect. AcLDL should increase the CE content of the cell, mevinolin should decrease the total cholesterol content of the cell and 58-035 should decrease the CE content but increase the FC content of the cell. Again the secretion in some of these studies was very low.

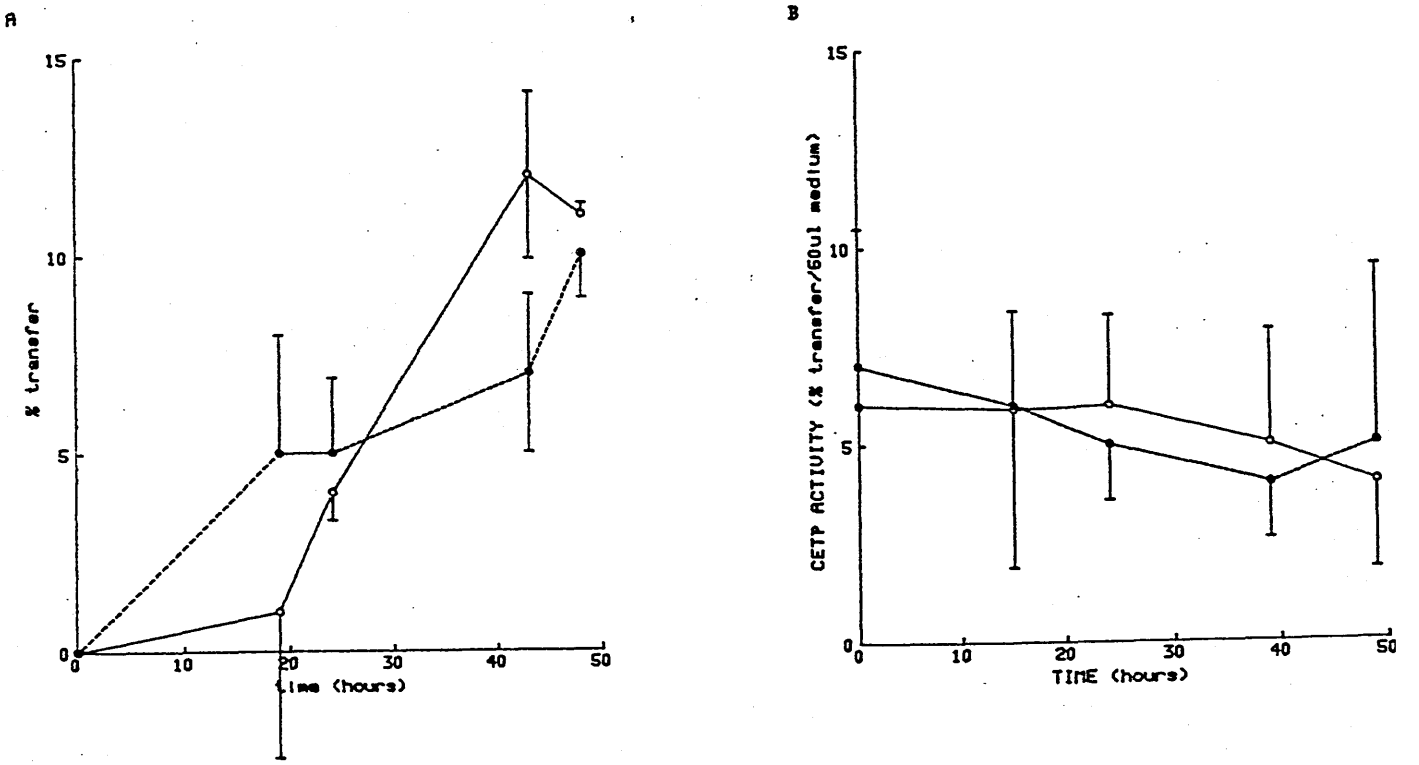


FIGURE 6.2 **CETP SECRETION BY MACROPHAGES - EFFECT OF PHORBOL MYRISTATE ACETATE (PMA) AND LIPOPOLYSACCHARIDE (LPS).** Human monocyte derived macrophages were isolated and cultured in 80cm^2 culture flasks for 8 days (PMA) or 12 days (LPS). The cells were washed with serum-free medium and then incubated with 20ml medium containing 40ng/ml PMA (A) or 500ng/ml LPS (B). Medium was sampled at 0, 19, 24, 43 and 48 hours (A) or 0, 16, 24, 39 and 49 hours (B). Sampled medium was frozen at -70°C and assayed for CETP activity using a sensitive CETP assay. (○) control cells, (●) treated cells.

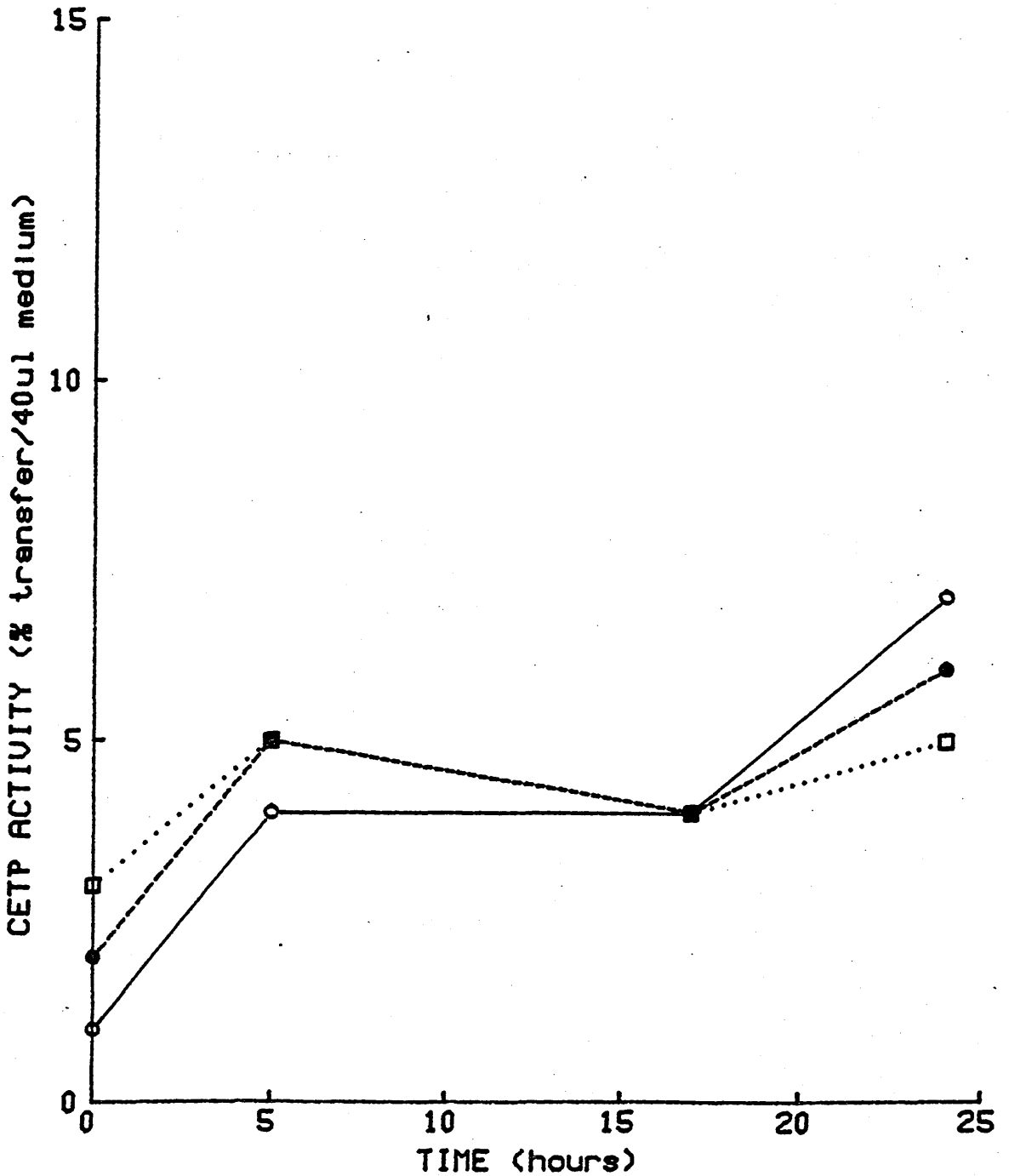


FIGURE 6.3 EFFECT OF INTERLEUKIN-1 (IL-1) ON CETP SECRETION BY HUMAN MONOCYTE DERIVED MACROPHAGES. Human monocyte derived macrophages were isolated and plated into 6 well plates. After 11 days in culture cells were washed with serum-free medium and then incubated in serum-free medium containing 0ng/ml (○), 10ng/ml (●) and 100ng/ml (□) IL-1. Conditioned medium was sampled at 0, 5, 17 and 24 hours and stored at -70°C. Medium was concentrated 4 fold using a Minicon concentrator and assayed for CETP activity.

Fig 6.4

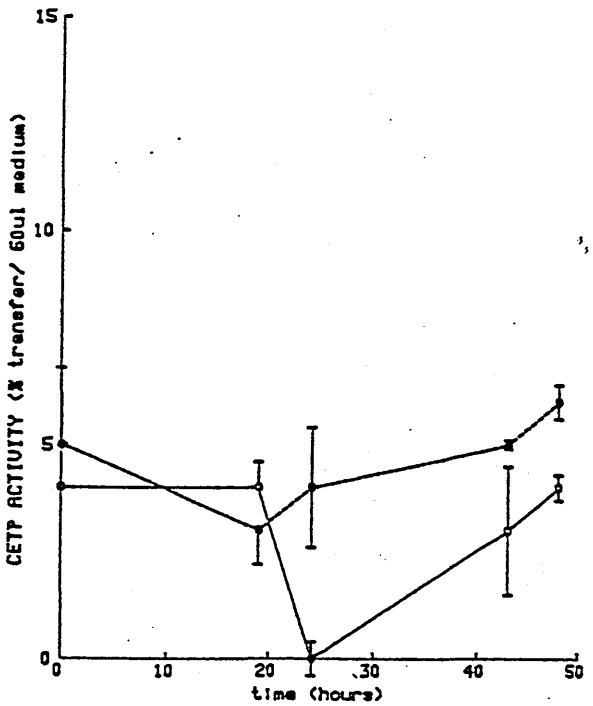


Fig 6.5

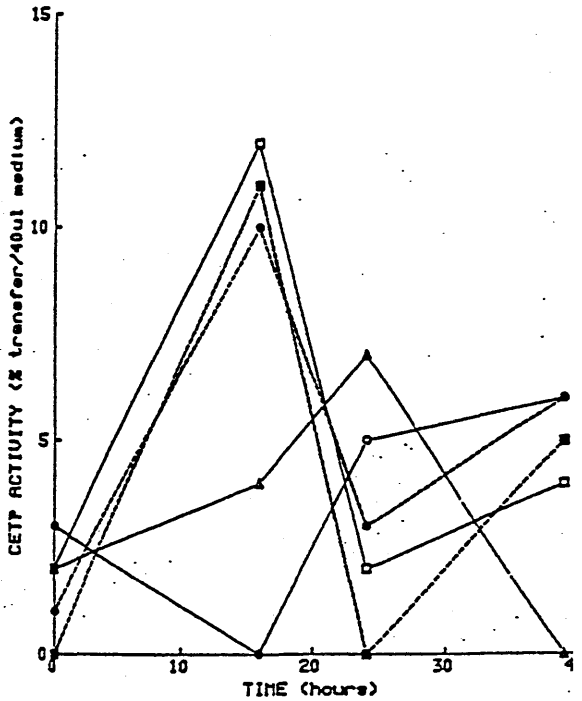


FIGURE 6.4 **THE EFFECT OF PMA ON CETP SECRETION BY J774 CELLS.** J774 cells were washed with serum-free medium and then cultured in 175cm^2 tissue culture flasks containing 40ml medium and cells at a density of 1x10^6 cell/ml. One flask contained control medium (○) and one flask contained medium with 40ng/ml PMA (●). At 0, 19, 24, 43 and 48 hours 1ml was sampled from each flask, the cells removed by centrifugation and the medium frozen at -70°C. CETP activity in the medium was assayed using a sensitive assay.

FIGURE 6.5 **THE EFFECT OF INCUBATION WITH ACETYL LDL, LDL, MEVINOLIN OR 58-035 ON CETP SECRETION BY J774 CELLS.** Five medium culture flasks were set up with J774 cells in serum-free medium at a density of 0.5x10^6 cells/ml. Flasks were; control (○), AcLDL 50μg/ml (●), mevinolin 100nM (□), 58-035 20μM (■) and LDL 50μg/ml (△). Samples (1ml) from each flask were taken at 0, 16, 24, 39 and 47 hours. Solution 4 (2ml) was added to each sample to bring the background density to 1.215g/ml and centrifuged for 3 hours in the Beckman TL100 centrifuge to remove the lipoproteins. Infranatant (2ml) was dialysed against Tris-saline buffer and then concentrated to 0.25ml using a Minicon concentrator and assayed for CETP activity.

Similar studies were carried out in the HepG2 cells and the CaCo-2 cells. HepG2 cells showed no response to PMA (Fig. 6.6). Of the treatments with AcLDL, LDL, mevinolin and 58-035, only LDL seemed to have a stimulatory effect at 24 hours (Fig.6.7). It was tested to see whether CaCo-2 cells could be stimulated to secrete more CETP with free fatty acid. With the cells used here, this effect was not seen (Fig. 6.8). It is possible that the CaCo-2 cells used here were not differentiated enough to have formed tight junctions and assembled their vectorial organisation.

In summary, although some effects were seen with IL-1 in human monocyte derived macrophages and with AcLDL, mevinolin and 58-035 in J774 cells the significance of these effects was difficult to determine since in general the amount of CETP activity secreted was very low and the errors in the CETP assay measurement were very large when the limit for sensitivity of the assay (about 5% transfer) was reached. Thus measurement of CETP activity in cell media was not a satisfactory way of investigating the control of CETP secretion. Had an antibody been available at the outset of this study then it could have been used to detect CETP in the medium by ELISA, dot-blotting or immunoelectrophoesis (if it was polyclonal).

3 Detection of CETP Messenger RNA levels in Cells

An alternative and more sensitive way of trying to ascertain the amounts of CETP secretion is to measure the amount of mRNA produced by these cells. The level of mRNA is not necessarily a direct measure of the amount of CETP secreted since all the mRNA may not be translated and all the protein may not be secreted. It will however give a good indication of whether gene transcription is being switched on due to regulation by certain effectors.

6.3.1 Probes

In order to detect mRNA from cells a probe which will hybridise to the CETP mRNA sequence is required. A 75 base pair oligonucleotide was selected from the human CETP cDNA sequence [Drayna, D. *et al* (1987)]. The oligonucleotide represents amino acid 126 to 150 of the CETP protein. The sequence was checked for homology to other known sequences by searching a computer database of structures, the 'Oligo' datafile (Genetics Department, Glaxo Group Reasearch Ltd, Greenford). The probe, GR91788X, was synthesised by Dr B. Coomber (Glaxo Group Research Ltd, Greenford) on a Biosearch oligonucleotide synthesiser. The properties of this probe and that of the 1581 base pair full length cDNA probe (gift from D. Drayna, Genentech, San Francisco) are shown in Table 6.2. A 25 base pair complementary primer, GR91789X, was also synthesised for use in radiolabelling the probe.

Probes could be labelled using the random priming method [Feinberg, A.P. & Vogelstein, B. (1984)] though in the case of GR91788X its specific primer GR91789X was used rather than random primers. Table 6.3 show the percentage incorporation and specific activities of all the probes used in these studies. They were all labelled to similar extents and to similar specific activities, The actin probe was used as a

Fig 6.6

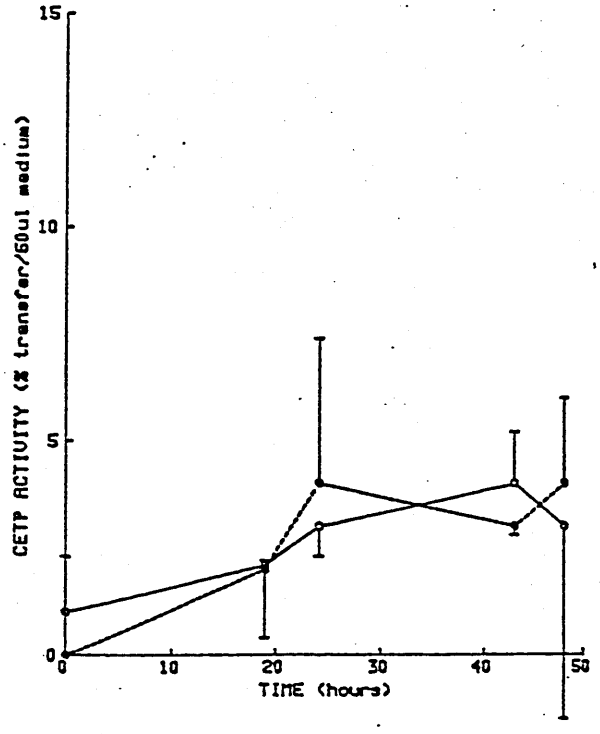


Fig 6.7

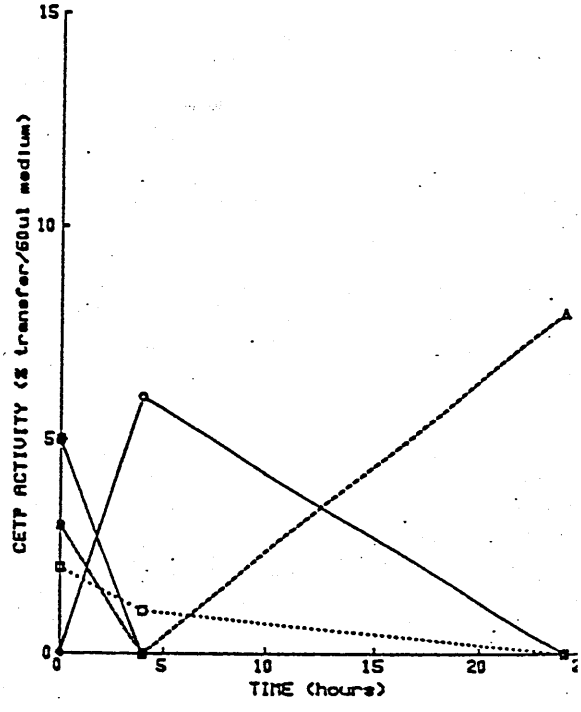


FIGURE 6.6 THE EFFECT OF PMA ON CETP SECRETION BY HEPG2 CELLS. Two 175cm^2 flasks of HepG2 cells were allowed to reach 90% confluence. The cells were washed with serum-free medium and then incubated in serum-free medium at 37°C (○) or serum-free medium containing 40ng/ml PMA (●). Samples (1ml) were taken at 0, 19, 24, 43 and 48 hours, centrifuged briefly to remove cell debris and frozen at -70°C. Samples were assayed for CETP activity using a sensitive assay.

FIGURE 6.7 THE EFFECT OF INCUBATION WITH ACETYL LDL, LDL, MEVINOLIN OR 58-035 ON CETP SECRETION BY HEPG2 CELLS. HepG2 cells were plated onto 6 well plates. When they were 90% confluent the cells were washed with serum-free medium and then incubated with serum-free medium, control (○) or serum-free medium containing AcLDL 50μg/ml (●), mevinolin 100nM (□), 58-035 20μM (■) and LDL 50μg/ml (△). Medium (1ml) was sampled at 0, 4 and 24 hours. Solution 4 (2ml) was added and the samples centrifuged for 3 hours in a Beckman TL100 tabletop centrifuge to remove the lipoproteins. Infranatants were dialysed against Tris-saline buffer, concentrated to 0.25ml and assayed for CETP activity.

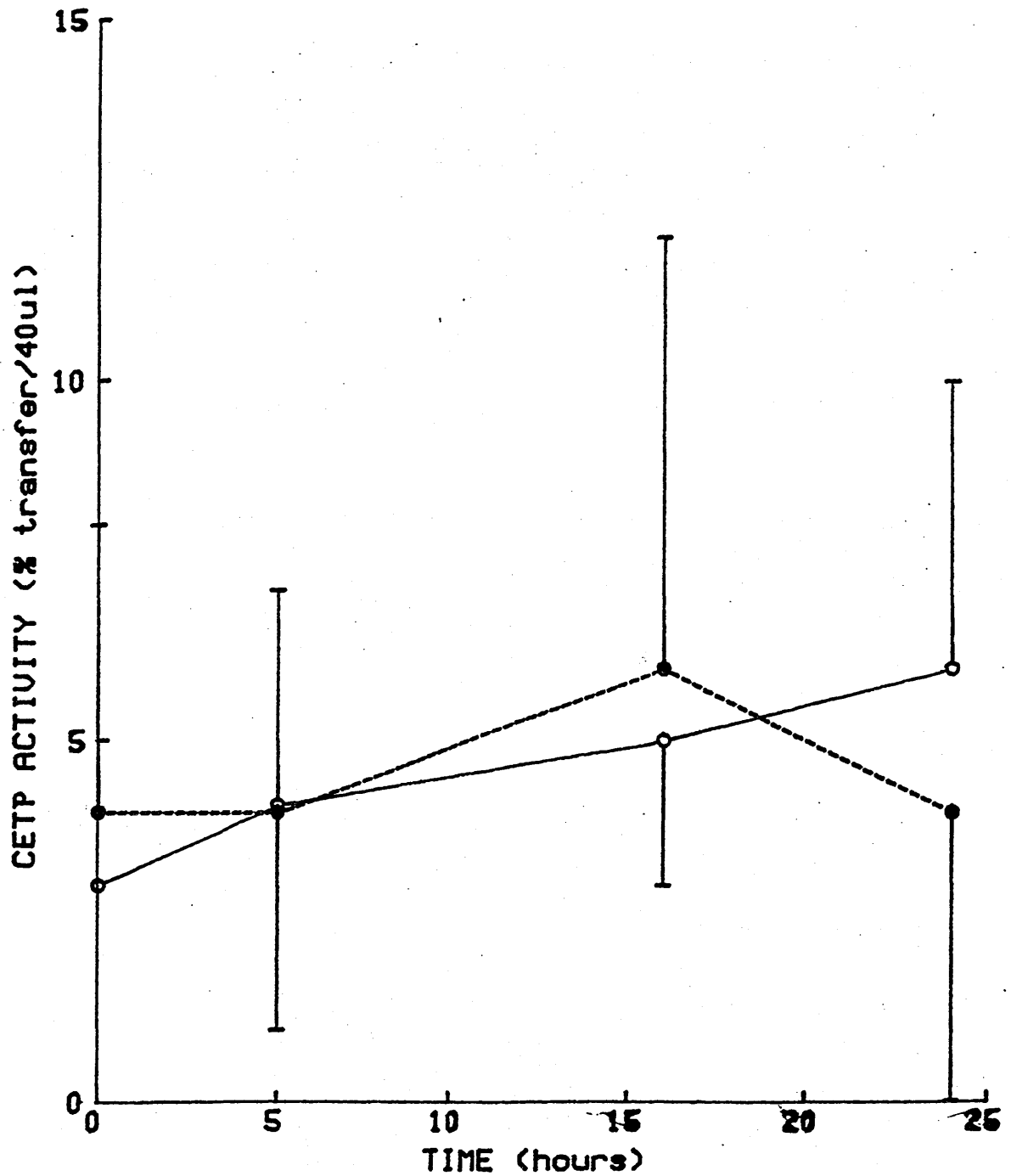


FIGURE 6.8 THE EFFECT OF FREE FATTY ACIDS ON THE SECRETION OF CETP BY CACO-2 CELLS. CaCo-2 cells were cultured to 2 weeks post confluence in Transwells. Each well was tested for its resistance and only cells with resistance >300Ω measured with a voltmeter were used. Cells were washed with serum-free medium and then incubated in serum-free medium (○) or serum-free medium containing 0.5mM sodium oleate (●), made as described in the methods section. Samples were taken at 0, 5, 16 and 24 hours, centrifuged to remove cell debris and frozen at -70°C. Medium was concentrated four fold using a Minicon concentrator and assayed for CETP activity.

	GR91788X	CETP cDNA
SOURCE	B. Coomber Glaxo	D. Drayna Genentech
CODING SEQUENCES	a.a. 126-150	a.a. 8-476 +178 untranslated
SIZE	75 bp	1581 bp
% C or G	54	54
SENSITIVITY OF DETECTION - THEMSELVES	0.5 pg	at least 0.5pg
SENSITIVITY OF DETECTION - EACH OTHER	10pg	0.1pg

TABLE 6.2 **PROBES FOR THE CETP GENE.** GR91788X was an oligonucleotide probe synthesised by B. Coomber (Glaxo Group Research Ltd, Greenford) and the CETP cDNA was a gift from D.Drayna (Genentech, San Francisco).

PROBE	n	INCORPORATION (%)	SPECIFIC ACTIVITY (dpm/μg)
GR91788X	7	62.7 +/- 16.2	2.2×10^9 +/- 0.6×10^9
CETP cDNA	6	63.5 +/- 15.4	2.3×10^9 +/- 0.4×10^9
ACTIN	2	54.5 +/- 6.4	1.4×10^9 +/- 0.1×10^9

TABLE 6.3 **INCORPORATION OF LABEL INTO AND SPECIFIC ACTIVITY OF PROBES USED FOR HYBRIDISATION.**

control as the mRNA for actin should be expressed in all cells, although when cells undergo division then actin message will increase as new protein is being synthesised, and confluent cells may not produce very much.

Once probes were available, suitable hybridisation conditions had to be found which would produce a specific signal for each probe. This was done by starting at the calculated T_M (38.6°C, see Appendix 2) for GR91788X and decreasing the temperature in small stages to find the best hybridisation conditions. The actin probe was provided commercially (Oncor) and Terry Baker (Glaxo Group Research Ltd, Greenford) advised of hybridisation conditions. The cDNA probe was provided by Dr D Drayna (Genentech) and I was advised of hybridisation conditions by Dr D Gaffney (Royal infirmary, Glasgow). The hybridisation conditions chosen are shown in Table 6.4.

6.3.2 Slot Blots

Initially slot blots of DNA and RNA were carried out to see whether hybridisation to the probe could be detected and to see how quantitative it was. The cDNA probe can be detected to 50pg and GR91788X to 0.5pg by hybridisation to GR91788X. Using the technique of slot blotting it was possible to detect 20 to 50 μg RNA from cells (Fig. 6.9) DNA from J774 and HepG2 cells and macrophages had strong signals upon hybridisation to GR91788X. The J774 total RNA signal could be seen clearly at 50μg loading and a signal with macrophage RNA at 100μg loading. There was only a faint hybridisation with HepG2 and macrophage total RNA at 50μg. There was no strong cross reactivity with yeast or calf liver rRNA which meant that message need not be purified as hybridisation can be carried out on total RNA without the rRNA interfering. However what was becoming clear from this blot was that hybridisation did not appear to be quantitative using this system e.g. 50μg macrophage RNA shows much less than 50% of the signal from the hybridisation to 100μg macrophage RNA. This may have been due either to unequal loading onto the slot blot or to the fact that the probe sometimes finds one area of the filter more accessible than another. This point was reemphasised in another blot (Fig 6.10) where hybridisation to untreated cell RNA was compared to RNA from cells treated with PMA. The signal was not proportional to the amount of RNA loaded so it was impossible to tell whether PMA had increased or decreased mRNA production. For J774 and HepG2 cells it did appear that the hybridisation signal was stronger for the RNA from treated cells. In this blot yeast rRNA did hybridise at 100μg.

6.3.3 RNA Gels

A more sensitive and quantitative way of detecting mRNA levels is a nuclease protection assay. In this assay probe is hybridised to mRNA in solution. The mix is then treated with S_1 nuclease which digests any single stranded DNA, so that the probe protects the region of interest. The product can be run on a gel and the product detected by autoradiography.

	+/- FORMAMIDE	HYBRIDISATION TEMPERATURE (°C)	WASHING TEMPERATURE (°C)
GR91788X	+	35	35
CETP cDNA	-	65	65
ACTIN	+	42	60

TABLE 6.4 HYBRIDISATION CONDITIONS FOR PROBES USED.

SAMPLE	GR91788X
GR91788X	+
CETP cDNA	+
CALF LIVER rRNA	-
YEAST rRNA	+
TOTAL RNA CaCo2	-
J774	-
macrophage	-
HepG2	-
mRNA CaCo2	-
J774	-
macrophage	-
HepG2	
TOTAL RNA RABBIT LIVER	-
mRNA RABBIT LIVER	-

TABLE 6.5 SUMMARY OF HYBRIDISATION RESULTS. Samples were prepared, electrophoresed on formaldehyde gels and Northern blotted onto Hybond-N as described in the methods. Membranes were probed with GR91788X and autoradiographed to detect any hybridising bands. + denotes hybridisation, - denotes no hybridisation.

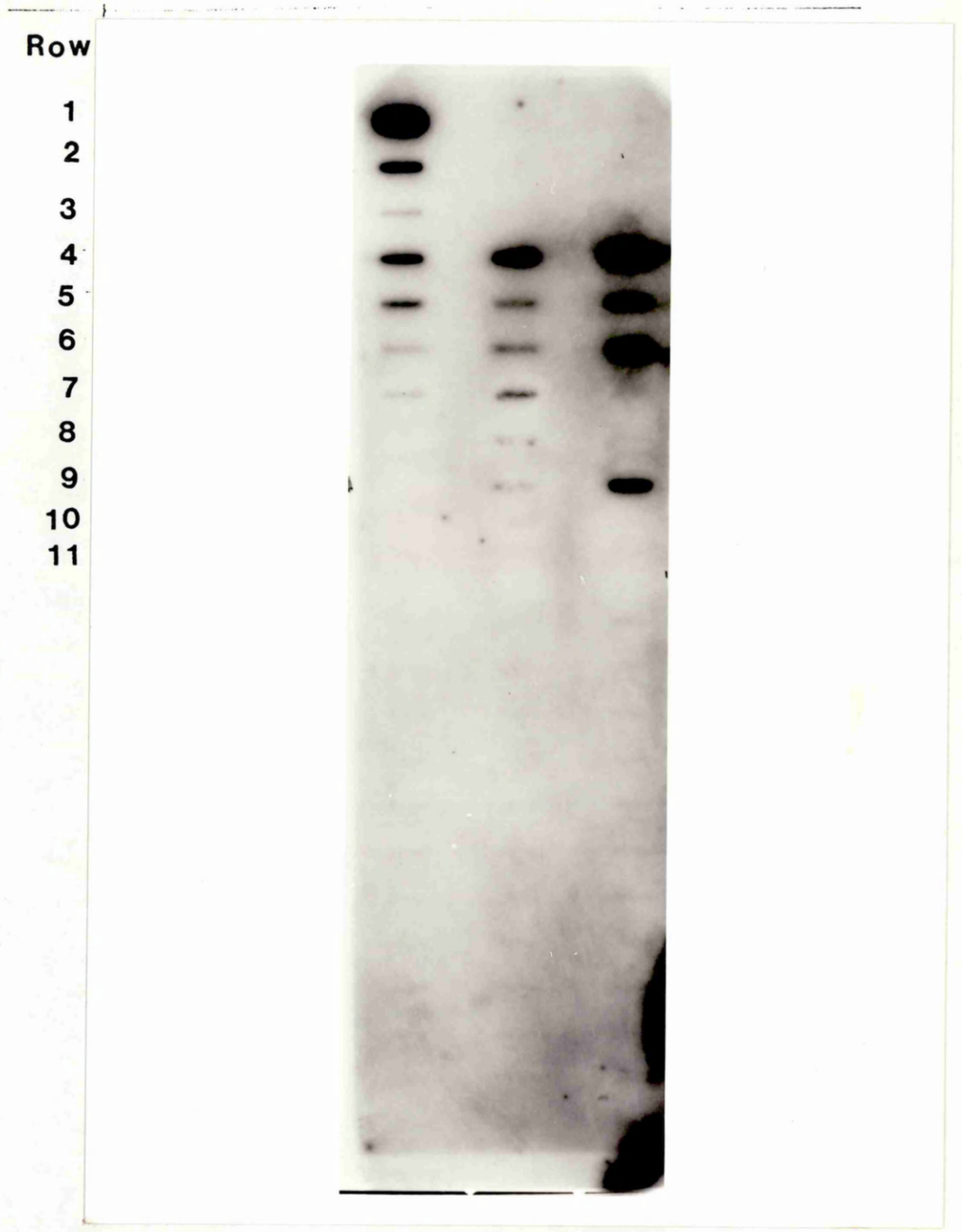


FIGURE 6.9 **SLOT BLOT OF J774, HEPG2 AND MACROPHAGE RNA AND DNA.** Sample preparation, blotting onto Hybond-N and hybridisation to GR91788X were carried out as described in the methods section. Rows 1 to 3: 10, 1 and 0.1pg GR91788X; Row 4: 5, 10 and 20μg J774 DNA; Row 5: 5, 10 and 20μg HepG2 DNA; Row 6: 5, 10 and 20μg macrophage DNA; Row 7: 20 and 50μg J774 RNA; Row 8: 20 and 50μg HepG2 RNA; Row 9: 20, 50 and 100μg macrophage RNA; Row 10: 5, 10 and 20μg calf liver ribosomal RNA; Row 11: 5, 10 and 20μg yeast rRNA.

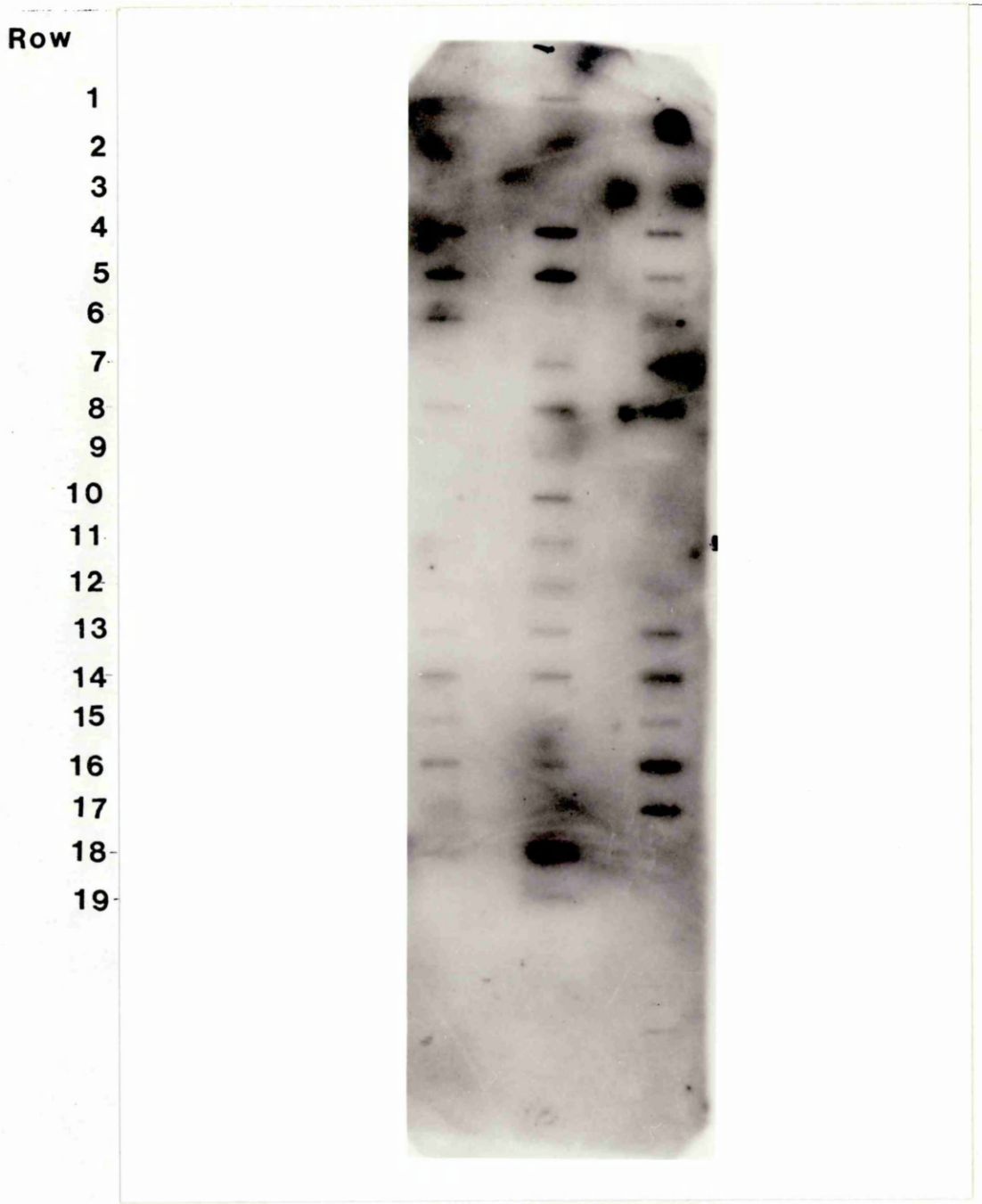


FIGURE 6.10 **SLOT BLOT OF RNA FROM CONTROL AND PMA TREATED J774 CELLS, HEPG2 CELLS AND MACROPHAGES.** RNA and DNA was prepared from cells cultured in the presence or absence of 40ng/ml PMA for 48 hours as described in the methods section. Samples were prepared, loaded onto a slot blot and hybridised to GR91788X. Row 1 to 3: 50, 5 and 0.5pg GR91788X; Row 4 and 5: 10μg DNA from control J774 cells, HepG2 cells and macrophages in duplicate; Row 6: control RNA J774 (50μg), HepG2 (30μg) and macrophage (90μg); Rows 7 to 12: the first two slots of each row are 20 and 50μg RNA, row 7 J774 control, row 8 J774 PMA-treated, row 9 HepG2 control, row 10 HepG2 PMA-treated, row 11 macrophage control, row 12 macrophage PMA-treated; Rows 13 to 17: 5, 10 and 20μg DNA, row 13 J774 control, row 14 J774 PMA-treated, row 15 HepG2 control, row 16 HepG2 PMA-treated, row 17 macrophage PMA-treated; Row 18 and 19: yeast and calf liver rRNA at 20 and 100μg

Prior to attempting this assay it is necessary to check that the mRNA from cells can be detected on a denaturing gel and that the probe will recognise the mRNA band of the correct length for CETP 1900 +/- 50 base pairs [Drayna, D. *et al* (1987)]. When total RNA is run on a formaldehyde gel the two ribosomal 28s and 21s bands can be seen after staining by ethidium bromide with the mRNA running as a smear in between. If the RNA is degraded then a lot of low molecular weight material can be seen.

When total RNA or mRNA from J774, HepG2 and CaCo-2 cells and from calf liver were run on formaldehyde gels, no hybridising band could be detected although the probe could detect itself and the cDNA (Table 6.5). Others have detected CETP message in human liver and other tissues but not in HepG2 or U937 cells [Drayna, D. *et al* (1987)]. There are two possible reasons why this might be the case. Either the RNA is being degraded or the CETP message is present at very low concentration. The extent of RNA degradation can usually be seen on the formaldehyde gels. Usually some smearing of degraded RNA can be seen below the lower ribosomal band but most of the RNA appears intact. However if the CETP RNA is particularly susceptible to degradation then it is possible that most of the CETP message is lost during the RNA preparation. If it is just that the CETP message is present in a very low amount then another gene message which should be present in high concentration (e.g. actin) should be easily detectable. For detecting CETP message from different animal tissues, Drayna *et al* [Drayna, D. *et al* (1987)] used 15μg of mRNA. The gels run here had only 20μg total RNA or 2μg mRNA. As the mRNA band is likely to be more diffuse after electrophoresis on an agarose gel then it may be more difficult to detect than on a slot blot. When a large amount of RNA (200μg) from HepG2, J774 and CaCo-2 cells was run on a formaldehyde gel and probed with an actin probe then for each lane two bands could be seen; one occurring between the 28s and 21s ribosomal RNA bands which corresponds to the actin message and a low molecular weight smear which probably represents fragments of degraded actin message. Thus it appears that both problems are occurring. Degradation of the RNA preparation is obviously taking place but despite this actin hybridisation can still be seen. It can be assumed that CETP message in the preparations will be being degraded. Since Drayna *et al* used large quantities of RNA check to detect message from tissues then the CETP message is probably present in low amounts. To improve detection on these Northern blots the RNA purification and electrophoresis conditions need to be improved so that degradation does not occur. One way of trying to improve detection of the signal is to amplify the message that is there using the polymerase chain reaction (PCR).

6.3.4 Polymerase Chain Reaction of CETP

Work was started to try and use PCR to amplify the CETP message. This would indicate whether the message was present and whether the failure to detect it was due to limits in sensitivity of detection. Suitable primers were selected such that a region of the CETP message that could be detected by GR91788X would be amplified.

Primers were chosen according to several criteria; they were 20 base pairs long, ended with a G or C, contained approximately GC:AT content and contained no runs or simple repeats. Two sense and two antisense primers were chosen with approximately 200 to 300 base pairs separating the sense primer from the antisense primer (Fig. 6.11). This 200 to 300 nucleotide stretch was complementary to the GR91788X probe. These primers were found to work successfully as primers using the CETP cDNA as a template (Fig. 6.12). Preliminary attempts to amplify this CETP sequence using RNA from J774, U937, HepG2 and CaCo-2 cells met with limited success. Only the J774 mRNA gave a weak band corresponding to the appropriate length for the message which would be expected with the primers used. Limitations on time precluded further investigations here. It will require a lot more work to find the optimal conditions for detecting the CETP message using this system.

SENSE PRIMERS	1	5' GAAGTATGGCTACACCACTG 3'
	2	5' GTCCATTGACTTCGAGATCG 3'
ANTISENSE PRIMERS	3	5' CGGCCATGATGTTAGAGATG 3'
	4	5' CTCCATCTGAAAGGATGCTG 3'

FIGURE 6.11 PRIMERS FOR AMPLIFICATION OF CETP CODING SEQUENCE BY THE POLYMERASE CHAIN REACTION.

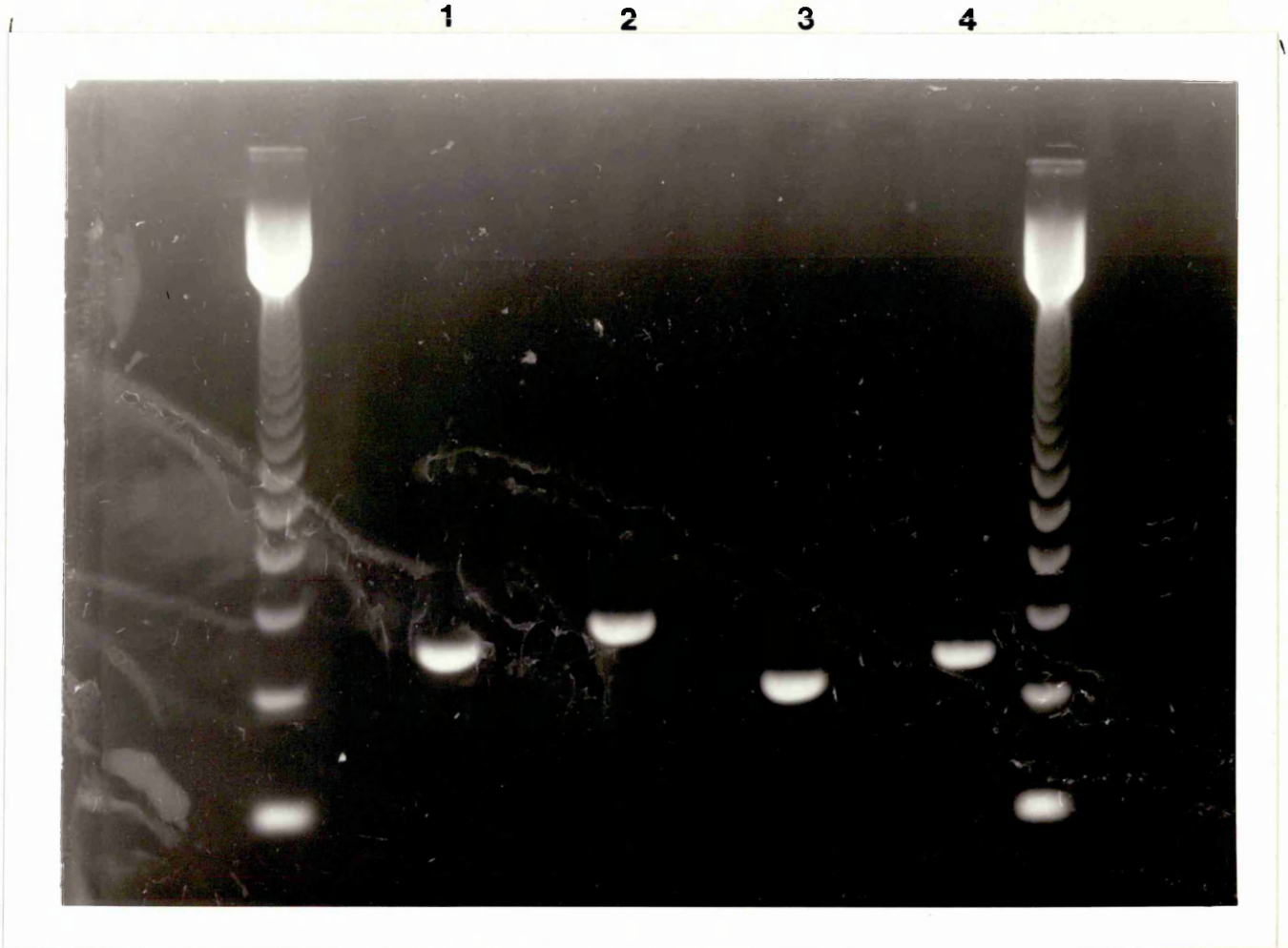


FIGURE 6.12 **POLYMERASE CHAIN REACTION USING CETP cDNA AS A TEMPLATE.** The polymerase chain reaction was carried out as described in the methods using 1ng cDNA as a template and primers 1 and 3, lane 1; primers 1 and 4, lane 2; primers 2 and 3, lane 3; primers 2 and 4, lane 4.

CHAPTER VII

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS OF THE CETP GENE.

1 Introduction

Genetic factors may play a role in the development of atherosclerosis. Restriction fragment length polymorphisms (RFLPs) have been used to study the variability in the genes of proteins associated with cholesterol metabolism. Mutations of genes can be detected when they alter the recognition sites for restriction enzymes. Digestion of DNA with these enzymes will result in different sizes of genomic DNA depending on the frequency of the restriction sites. The fragments can be separated by gel electrophoresis and fragments from the gene of interest can be detected by their hybridisation to a gene probe. If variation can be detected in candidate genes then it is possible to investigate whether this genetic variation is related to an individual's phenotype. RFLPs may be at the site of the mutation, or more likely, may be linked to the site of the mutation.

There appears to be a negative correlation between plasma HDL cholesterol levels and plasma CETP activity [Tollefson, J.H. *et al* (1988)] and animals which have low HDL levels have high CETP activities and *vice versa* [Ha, Y.C. & Barter, P.J. (1982), Chapman, M.J. (1986)]. Within the human species, individuals with extremely high HDL cholesterol, hyperalphalipoproteinaemics, have low or absent CETP activity [Koizumi, J. *et al* (1985), Kurasawa, T. *et al* (1985)]. Since HDL levels seem to be related to CETP activity it was decided to investigate whether variation in the CETP gene (as detected by RFLPs), was related to plasma levels of the enzyme and whether this also might be reflected in plasma HDL levels. The cDNA of the human CETP gene has been cloned [Drayna, D. *et al* (1987)] and was made available by Dr D Drayna, Genentech. Two RFLPs of the CETP gene had already been detected using the restriction enzyme TaqI [Drayna, D. & Lawn, R. (1987)]. The TaqIA polymorphism can be detected as fragments of two lengths; A_1 7.5kb and A_2 9.0kb, occurring with frequencies of 0.88 and 0.12 respectively. The TaqIB polymorphism can be detected as fragments of B_1 4.4kb and B_2 5.3kb of frequencies 0.52 and 0.48 respectively. The frequencies for both these polymorphisms were measured in a population of 32 subjects.

2 Polymorphisms of the CETP Gene

A battery of restriction enzymes were tested to see whether any further RFLPs of the CETP gene could be detected. A group of 10 individuals were chosen from a population of 65 individuals selected from an opportunistic screening programme. These

individuals were those with the five highest and the five lowest HDL levels. If any polymorphism were to occur at a reasonable frequency, i.e. greater than 0.1, then it would probably be detected within this small group.

The 1581bp cDNA CETP clone was initially used to hybridise to the fragments produced by the enzyme. The CETP gene was not polymorphic for the restriction enzymes AluI, ApaI, BamHI, BanII, BglI, BglII, BstEII, ClaI, DraI, EcoRI, EcoRV, HaeIII, HincII, HindIII, HinfI, HhaI, HpaI, KpnI, PstI, PvuII, RsaI, SalI, SmaI, SstII, XbaI and XhoI. The CETP gene was however polymorphic for the restriction enzyme StuI [Freeman, D. *et al* (1989)]. This was detected as a pattern of three invariant bands; 5.8kb, 4.8kb and 3.4kb, and the presence (S_1) or absence (S_2) of a 4.0kb band. Detection of this 4.0kb band was difficult. To try and improve detection a shorter, 732bp, fragment of the CETP cDNA probe was used (work carried out by Dr Dairena Gaffney, Royal Infirmary, Glasgow). This was obtained by digesting the 1581bp probe with PstI and PvuII restriction enzymes and corresponds to bp 677 to 1409 of the published sequence [Drayna, D. *et al* (1987)]. The StuI blots from the 10 individuals were reprobed with this smaller probe and it was found that two allelic fragments at 4.0 and 4.3kb could be detected (Fig. 1), in addition to the invariant 3.4kb band. It thus appears that the S_1 allele at 4.0kb was being detected previously but that the hybridisation signal of the larger probe to the 4.3kb band was not strong enough to be seen. This may be because the three invariant bands at 5.8, 4.8, and 3.4kb, obtained when probing with the large 1581bp probe, hybridised strongly to the labelled probe while the 4.0 and 4.3kb bands hybridised only weakly. The small probe did not hybridise to the larger invariant bands and therefore the 4.0 and 4.3kb bands could hybridise more probe and a stronger signal was discerned. Once the polymorphism was detected the whole population of 65 individuals was screened. From the 42 results obtained (the other results were ambiguous) the frequency of the S_1 (4.0kb) allele was calculated to be 0.92 and the frequency of the S_2 (4.3kb) allele was 0.08. Only individuals with the S_1 band alone and both bands together were detected. These were classified as S_1S_1 homozygotes and S_1S_2 heterozygotes respectively.

Mendelian codominant inheritance was demonstrated in one family (Fig. 1). The first six tracks were probed with the 1581bp probe. As can be seen it is difficult to determine which individuals express the 4.0kb band. The mother (A in Fig. 1) is S_1S_2 and the father (B) is S_1S_1. The son (C) has inherited the S_1S_1 genotype and the daughter (D) has inherited the S_1S_2 genotype.

The TaqIA and B polymorphisms were also detected in this population. It was found that using a 362 bp probe which corresponded to amino acids 8 to 128 in the mature protein, obtained by EcoRI and PvuII double digestion of the 1581bp CETP cDNA, that the detection of the TaqIB polymorphism was much clearer. Dr Dairena Gaffney, Royal Infirmary, Glasgow, produced the 362bp probe and carried out any reprobing of ambiguous blots.

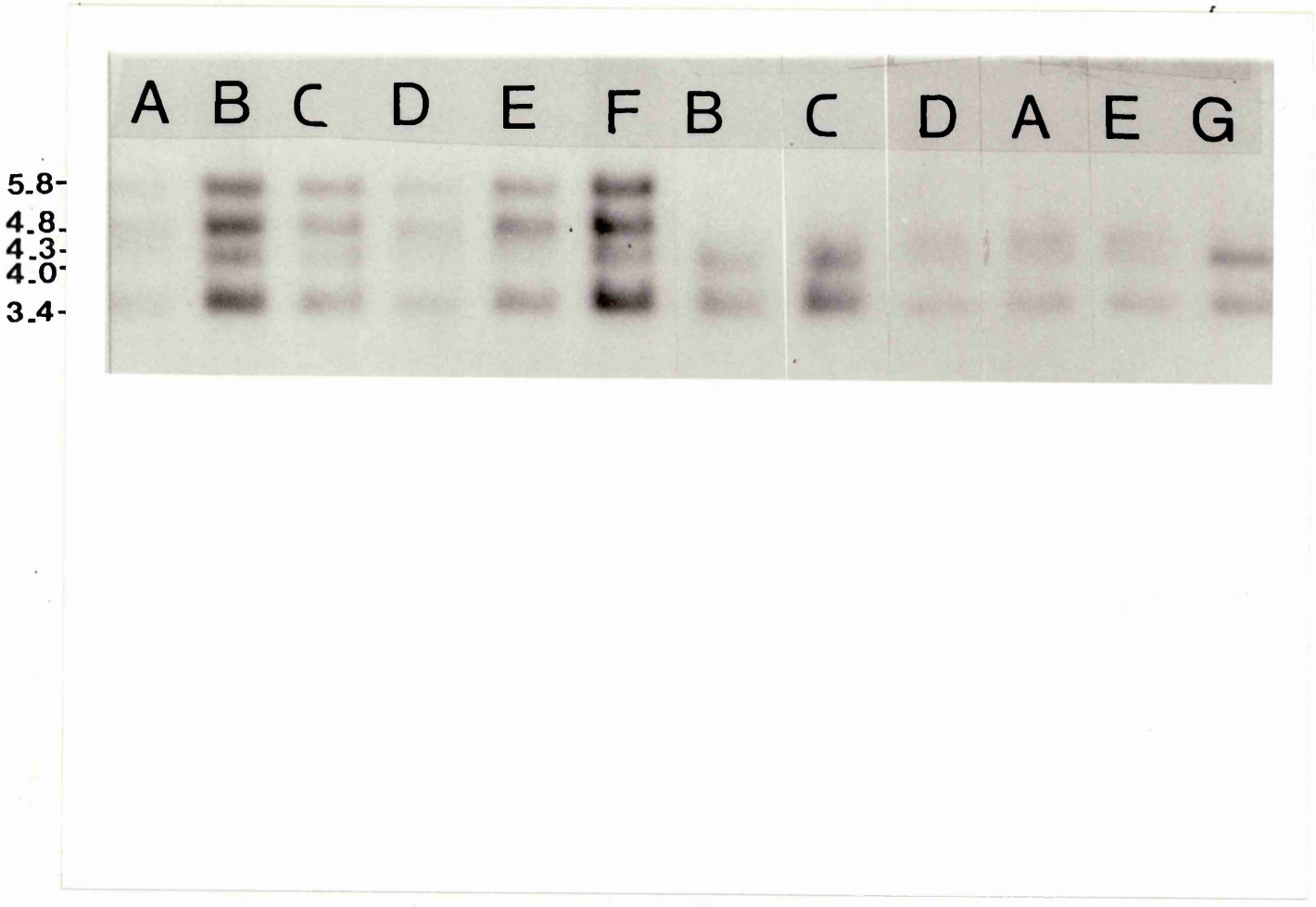


FIGURE 7.1 **MENDELIAN INHERITANCE OF THE STU I RFLP.** Human DNA was digested with StuI according to the manufacturer's instructions and run on an agarose gel. The first six tracks were hybridised with labelled 1581bp CETP probe and the last six tracks were probed with the 732bp subfragment. A = mother, B = father, C = son, D = daughter, E,F,G = unrelated individuals.

3 A Study to Investigate the Relationship of RFLPs of the CETP Gene to Plasma Lipid Parameters

7.3.1 Strategy

The aim of this study was to examine the possibility that variation in the CETP gene may be associated with alterations in the activity of plasma CETP and plasma HDL cholesterol levels. Plasma LCAT activity was also measured since the CETP and LCAT genes are in close proximity on chromosone 16; 16q21 and 16q22 respectively. It is possible the variation detected in the CETP gene may be marking a linked mutation in the LCAT gene. Besides these two enzyme activities, plasma total and HDL cholesterol were measured.

Fifty-six individuals were selected from an opportunistic screening programme in Glasgow on the basis of their HDL levels. Normotryglyceridaemic (<3.0mM) individuals with low HDL levels (<1mM) or high HDL levels (>2.5mM for females and >2.0mM for males) were selected. Plasma total cholesterol, HDL cholesterol, CETP activity and LCAT activity were measured. The individuals were also typed for the TaqI and StuI polymorphisms of the CETP gene and the MspI and PstI polymorphisms of the apoAI gene [Coleman, R.T. *et al* (1986)]. RFLPs of the apoAI gene were studied since apoAI is a component of HDL and a cofactor of LCAT and it was necessary to see whether any trends in the lipid parameters were associated with variation in this gene. It would also give an indication of gross disturbances of lipid parameters or genes in this population. No polymorphisms of the LCAT gene have yet been detected. The lengths of the polymorphic fragments detected by hybridisation to CETP or apoAI gene probes are shown in Table 7.1. The genotype frequencies are also shown. The allele frequencies for the TaqIB polymorphism differ from those obtained by others [Drayna, D. & Lawn, R, (1987)]. This may be due to differences in the selected populations as both sample sizes are rather small. All statistical analyses were carried out by Marion Chatfield, Glaxo Group Research Ltd, Ware.

7.3.2 Cholesterol Levels

Total cholesterol levels were measured in 48 individuals from the population and were found to be normally distibuted. For each polymorphism, the number, mean, standard deviation and range of the cholesterol measurements is given for each genotype within the polymorphism (Table 7.2). T-test and analysis of variance were then used to test whether total cholesterol levels differed between the genotypes. There was no significant difference between the total cholesterol levels of each genotype group for any of the polymorphisms.

POLYMORPHISM	FRAGMENT LENGTHS (kb)		GENOTYPE FREQUENCY			ALLELE FREQUENCY	
TaqIA	A_1	A_2	A_1A_1	A_1A_2	A_2A_2	A_1	A_2
	7.5	9.0	43	6	0	0.94	0.06
TaqIB	B_1	B_2	B_1B_1	B_1B_2	B_2B_2	B_1	B_2
	4.4	5.3	26	19	11	0.63	0.37
StuI	S_1S_1	S_1S_2	S_1S_1		S_1S_2	S_1	S_2
	4.3	4.0	34		8	0.90	0.10
PstI	P_1	P_2	P_1P_1	P_1P_2	P_2P_2	P_1	P_2
	2.2	3.3	43	6	0	0.94	0.06
MspI	M_1	M_2	M_1M_1	M_1M_2	M_2M_2	M_1	M_2
	0.67,1.08	1.75	42	11	0	0.90	0.10

TABLE 7.1 **POLYMORPHISMS OF THE CETP AND APO AI GENES.** The TaqIA, TaqIB and StuI polymorphisms are linked to the CETP gene. PstI and MspI polymorphisms are linked to the apo AI gene. For TaqIA and TaqIB number of individuals n=56, PstI n=49, MspI n=53 and for StuI n=42. In some instances technical difficulties precluded satisfactory assignment of genotype.

POLYMORPHISM	STATISTIC	TOTAL CHOLESTEROL (mM)			PROBABILITY OF SIMILARITY
TaqIA	GENOTYPE	A_1A_1	A_1A_2		
	No.	42	6		
	MEAN	6.18	5.48		
	STD. DEV.	1.10	0.88		0.15
	RANGE	4.25,8.70	3.85,6.45		
TaqIB	GENOTYPE	B_1B_1	B_1B_2	B_2B_2	
	No.	25	15	8	
	MEAN	6.39	5.64	6.01	
	STD. DEV.	1.01	1.20	0.93	0.11
	RANGE	4.80,8.70	3.85,8.40	5.00,8.00	
StuI	GENOTYPE	S_1S_1	S_1S_2		
	No.	34	7		
	MEAN	6.15	5.96		
	STD. DEV.	1.23	0.74		0.70
	RANGE	3.85,8.70	5.05,7.15		
PstI	GENOTYPE	P_1P_1	P_1P_2		
	No.	42	6		
	MEAN	6.08	6.19		
	STD. DEV.	1.07	1.34		0.81
	RANGE	4.25,8.70	3.85,7.50		
MspI	GENOTYPE	M_1M_1	M_1M_2		
	No.	37	9		
	MEAN	6.07	6.22		
	STD, DEV.	1.14	1.06		0.72
	RANGE	3.85,8.70	4.80,8.00		

TABLE 7.2 **TOTAL CHOLESTEROL LEVELS AND POLYMORPHISMS.** For each polymorphism the number, mean, standard deviation and range of total cholesterol (mM) is given. T-tests and analysis of variance were used to test whether total cholesterol differed between genotypes.

7.3.3 HDL Cholesterol

HDL levels were measured before and after recall and the average used for analysis. There were 39 individuals in the low HDL group (28 males and 11 females) and 17 individuals (7 male and 10 female) in the high HDL group. Table 7.3 shows for each polymorphism the number of individuals of a particular genotype in the high or low HDL group. A Fisher's Exact test was carried out ot test whether the distribution of genotypes in the high and low HDL groups differed between the two groups. For the TaqIB polymorphism the difference in distribution of individuals between genotypes B_1B_1, B_1B_2 and B_2B_2 in the high and low HDL groups was significant (P=0.0014). Class B_1B_1 has 62% of the individuals in the low HDL group but only 12% of the patients in the high HDL group. There was no significant difference (P>0.05) between the proportion of individuals of each genotype between the two HDL groups for the other polymorphisms.

HDL levels are affected by sex; females tend to have higher HDL levels than males; thus it is important to look at the sexes separately. Table 7.4 divides the TaqIB data into males and females. The number of individuals in each class of the polymorphism (and the percentage of the total for the group) are given for high and low HDL groups and shown for males and females separately. The difference between high and low HDL groups in the percentage of individuals occuring in each polymorphism class is calculated for males and for females. It can be seen that the differences between high and low HDL groups are similar for both males and females. The numbers in each group are too small for this to be tested statistically. Since the observation that a large percentage of low HDL individuals are B_1B_1 and a low percentage of high HDL individuals are B_1B_1 is seen both for males and for females the data can be combined.

There was no difference in cholesterol level (P=0.12) or LCAT activity (P=0.48), using t-tests, between the high and low HDL groups. CETP activity did significantly differ (P=0.006), using a Wilcoxon signed rank test, between the two groups; median CETP activity in the low group was 0.15 %t/μgLPDS/2.5 hours and median CETP activity in the high HDL group was 0.11 %t/μgLPDS/2.5 hours.

7.3.4 Plasma CETP Activity and LCAT Activity

CETP activity did not appear to be normally distributed in this population. CETP activity is summarised for each genotype for each polymorphism in Table 7.5. The Wilcoxon signed rank test or Kruskal Wallis test were used to examine whether CETP activity differed between genotypes for each polymorphism. There was a significant difference between the classes of the TaqIB (P=0.01) polymorphism and a nearly significant (P=0.057) difference between the classes of the StuI polymorphism.

POLYMORPHISM		LOW HDL GROUP No(%)	HIGH HDL GROUP No(%)	PROBABILITY OF SIMILARITY IN PERCENTAGES
TaqI	A_1A_1	35(91)	8(80)	0.59
	A_1A_2	4(9)	2(20)	
	B_1B_1	24(62)	2(12)	
	B_1B_2	10(26)	9(53)	0.0014
	B_2B_2	5(12)	6(35)	
StuI	S_1S_1	27(79)	7(88)	1.0
	S_1S_2	7(21)	1(12)	
PstI	P_1P_1	33(85)	10(100)	0.32
	P_1P_2	6(15)	0(0)	
MspI	M_1M_1	30(81)	12(75)	0.72
	M_1M_2	7(19)	4(25)	

TABLE 7.3 **DISTRIBUTION OF INDIVIDUALS BETWEEN HIGH AND LOW HDL GROUPS.** For each polymorphism the number of individuals of a particular genotype is given for high (HDL cholesterol >2.0mM for men, >0.25mM for women) or low HDL (HDL cholesterol <1.0mM) groups. This is also expressed as a percentage of the total number of individuals in that group. A Fisher's Exact test was used to test whether the distibution of genotypes in high and low HDL groups differed between the groups.

POLYMORPHISM	MALES LOW HDL	MALES HIGH HDL	DIFF %	FEMALES LOW HDL	FEMALES HIGH HDL	DIFF %
	NO. (%)	NO. (%)		NO. (%)	NO. (%)	
B_1B_1	17 (61)	0 (0)	-61	7 (64)	2 (20)	-44
B_1B_2	7 (25)	4 (57)	32	3 (27)	5 (50)	23
B_2B_2	4 (14)	3 (43)	29	1 (9)	3 (30)	21

TABLE 7.4 **DISTRIBUTION OF INDIVIDUALS BETWEEN HIGH AND LOW HDL GROUPS FOR MALES AND FEMALES.** The data from Table 7.3 is shown for the TaqIB polymorphism. The data for males and females are displayed separately. The difference in the percentage of individuals in the high and low HDL groups is given for each class of the polymorphism.

POLYMORPHISM	STATISTIC	CETP ACTIVITY (%t/μg LPDP protein)			PROBABILITY OF SIMILARITY
TaqIA	GENOTYPE	A_1A_1	A_1A_2		
	No.	43	6		
	MEDIAN	0.157	0.133		
	QUARTILES	0.114,0.204	0.127,0.141		0.63
	RANGE	0.069,0.590	0.124,0.235		
TaqIB	GENOTYPE	B_1B_1	B_1B_2	B_2B_2	
	No.	26	19	11	
	MEDIAN	0.181	0.124	0.139	
	QUARTILES	0.140,0.209	0.110,0.157	0.070,0.162	0.010
	RANGE	0.075,0.590	0.078,0.235	0.056,0.227	
StuI	GENOTYPE	S_1S_1	S_1S_2		
	No.	34	8		
	MEDIAN	0.145	0.210		
	QUARTILES	0.123,0.199	0.160,0.231		0.057
	RANGE	0.069,0.445	0.070,0.590		
PstI	GENOTYPE	P_1P_1	P_1P_2		
	No.	43	6		
	MEDIAN	0.150	0.148		
	QUARTILES	0.122,0.204	0.140,0.164		0.65
	RANGE	0.069,0.590	0.085,0.180		
MspI	GENOTYPE	M_1M_1	M_1M_2		
	No.	42	11		
	MEDIAN	0.143	0.139		
	QUARTILE	0.110,0.200	0.088,0.162		0.28
	RANGE	0.056,0.590	0.069,0.180		

TABLE 7.5 **PLASMA CETP ACTIVITY AND POLYMORPHISMS.** For each polymorphism the number, median, quartiles and range of CETP activity (% transfer/μg LPDP protein) are given. The Wilcoxon signed rank test and Kruskal Wallis test were used to test whether CETP activity differed between genotypes. Quartiles are defined such that the % of observations below them is 25 and 75 respectively.

Plasma LCAT activity was log normally distributed in this population. A summary of LCAT activity for each genotype for each polymorphism is shown in Table 7.6. Using t-tests and analysis of variance it was observed that only in the case of the MspI polymorphism of the apoAI gene did LCAT activity significantly differ between genotypes M_1M_1 and M_1M_2 (P=0.003).

7.3.5 Summary

This study set out to examine whether genetic variation at the CETP gene may have an influence on plasma HDL cholesterol levels. The relationship between polymorphisms in the CETP gene, apoAI gene and HDL levels was investigated in 56 unrelated individuals with high or low HDL levels. RFLPs of the apoAI gene were included to check for disturbance in the apoAI gene [Ordovas, J.M. *et al* (1986), Kessling, A.M., Horsthemke, B. & Humphries, S.E. (1985)] which may affect HDL levels. The apoAI polymorphisms were not related to HDL cholesterol or plasma CETP activity in this population but there was a significant relationship between the MspI polymorphism and plasma LCAT activity. M_1M_1 individuals had lower plasma LCAT activities than M_1M_2 individuals. Since the d>1.215g/ml fraction of plasma used to assay the enzyme will contain some apoAI stripped from the individuals' HDL during ultracentrifugation, this relationship may be merely reflecting the ability of the individuals' apoAI to act as a cofactor for LCAT. However a large excess of exogenous apoAI is added to the LCAT assay and should overcome any of this variability. This observation needs to be confirmed in a larger sample population.

CETP may have a strong influence on HDL levels. It was found that the low HDL group of individuals had a 40% higher median CETP activity than the high HDL group. While the TaqIA polymorphism of the CETP gene showed no relationship to HDL cholesterol or CETP activity, the TaqIB polymorphism was significantly related to both these parameters. Individuals with the B_2 allele were more likely to have high HDL levels and low CETP activity. The StuI polymorphism showed a near significant link with CETP activity but not with HDL cholesterol. Since the TaqIB polymorphism can be detected by the 362bp probe (amino acids 8 to 128) then this indicates that the polymorphism is towards the 5 prime end of the gene. Since it appears from the genomic sequence that the CETP gene is made up of many short exons interrupted by large introns [Agellon, L.B. *et al* (1989)] it is possible thet the variable site occurs within an intron. It is not known whether this effect on CETP activity is an effect on protein mass or CETP inhibitor. The whereabouts of the CETP inhibitor gene is unknown. Since none of the polymorphisms in the CETP gene were associated with LCAT activity it is unlikely that the results can be attributed to a change in the LCAT gene which is in linkage disequilibrium with a CETP polymorphism. There has been a report [Borresen, A-L. *et al* (1987)] where an effect of haptoglobin subtypes on serum HDL levels was demonstrated. They suggested that this result may be due to linkage to LCAT. From the data presented here, it is possible that the result is due to linkage to the CETP gene.

POLYMORPHISM	STATISTIC	LCAT ACTIVITY (pmol/hr/μgLPDP protein)			PROBABILITY OF SIMILARITY
TaqIA	GENOTYPE	A_1A_1	A_1A_2		
	No.	43	6		
	GEOMETRIC MEAN	0.366	0.326		
	RANGE	0.186-0.962	0.207-0.697		0.54
	LOG MEAN	-1.01	-1.12		
	LOG STD. DEV.	-5.53	-5.66		
TaqIB	GENOTYPE	B_1B_1	B_1B_2	B_2B_2	
	No.	26	19	11	
	GEOMETRIC MEAN	0.367	0.352	0.427	
	RANGE	0.186-0.686	0.207-0.697	0.191-0.962	0.43
	LOG MEAN	-1.00	-1.04	-0.85	
	LOG STD. DEV.	-5.58	-5.30	-5.39	
StuI	GENOTYPE	S_1S_1	S_1S_2		
	No.	34	8		
	GEOMETRIC MEAN	0.376	0.302		
	RANGE	0.204-0.697	0.186-0.737		0.15
	LOG MEAN	-0.98	-1.20		
	LOG STD. DEV.	-5.93	-5.50		
PstI	GENOTYPE	P_1P_1	P_1P_2		
	No.	43	6		
	GEOMETRIC MEAN	0.358	0.368		
	RANGE	0.191-0.737	0.186-0.962		0.89
	LOG MEAN	-1.03	-1.00		
	LOG STD. DEV.	-5.54	-5.39		
MspI	GENOTYPE	M_1M_1	M_1M_2		
	No.	42	11		
	GEOMETRIC MEAN	0.337	0.490		
	RANGE	0.186-0.686	0.286-0.962		0.0033
	LOG MEAN	-1.09	-0.71		
	LOG STD. DEV.	-5.57	-5.58		

TABLE 7.6 **PLASMA LCAT ACTIVITY AND POLYMORPHISMS.** For each polymorphism the number, mean and standard deviation of the logged data is given. The geometric mean and range are also shown. T-tests and analysis of variance were used to test whether LCAT activity logged differed between genotypes.

A report linking the TaqIB allele with high HDL levels and high apoAI levels was published [Kondo, I. *et al* (1989)]. The study carried out here confirms some of their observations and implicates the involvement of CETP by direct measurement of plasma CETP activity.

There are limitations on interpreting data of this type. The population size, n=56, is small and when rare genotypes are studied then the number of individuals in a particular genotype class is low. Since the HDL distribution was deliberately biased in order to try and see an effect, this also made statistical analysis difficult. Ideally this study should be extended to cover a larger sample population with a normal HDL distribution and an equal representation of males and females.

CHAPTER VIII

DISCUSSION

1 CETP Assay

Assaying CETP can be a complicated process. Activity can be measured in two ways either by exchange or as net mass transfer. It is also possible to measure the protein mass of the enzyme. There are factors other than mass e.g. substrate lipoproteins, LCAT, lipoprotein lipase and CETP inhibitor, all of which can influence observed CETP activity to a greater or lesser extent. Thus it is important to decide what measurement is actually required. For studies on the control of the secretion of CETP protein or synthesis of CETP it is relevant to look at protein mass. However for experiments on changes in plasma levels of CETP activity the situation is more complicated. Is it more relevant to measure CETP activity *per se* or CETP activity in the presence of inhibitor? Is it comparable to measure CETP activity in different individuals using their endogenous lipoproteins or is it better to use exogenous lipoproteins standardised for a set of measurements and compare activities without the influencing effect of variation in lipoprotein concentration? The answers to these questions will depend greatly on what the experiment is designed to determine. If for example a drug treatment is known to alter lipoprotein composition but is being studied for its effect on CETP activity rather than on protein concentration then the influence of the change in lipoprotein composition may also be taken into account by using endogenous lipoproteins in the assay. If however a measurement of the expression of CETP activity is required i.e. net of CETP plus inhibitor protein, then it may be more reasonable to use exogenous lipoproteins. Changes in plasma CETP activity may be due to changes in protein mass, inhibitor protein or activation of the enzyme. Ideally, to dissect the situation out, measurement of protein mass, inhibitor mass and exchange or net transfer activity should be carried out. This is especially important when looking at control mechanisms involved in regulating plasma CETP activity as so many things can affect CETP activity. Unfortunately the tools to carry out these assays have not been available.

In this thesis CETP exchange activity was determined using exogenous lipoproteins which measured activity in the presence of inhibitor. The activity measured demonstrated the properties reported for CETP in the literature. It was decided that this activity was probably the most relevant and most practical given the tools available for looking at changes in plasma CETP activity but would allow no conclusions to be drawn as to whether any change in measured activity resulted from changes in enzyme mass or an

alteration of its activity. It is possible to remove inhibitor using a phenyl Sepharose column but this was not feasible for a large number of samples and was only attempted with the cell secretion studies when little CETP activity could be measured and it was suspected that CETP inhibitor was being secreted too. This exchange assay was able to allow processing of large numbers of samples relatively easily and any change in plasma CETP activity was readily detectable. The problem was that there were limitations on the interpretation of changes in activity.

Another problem arose from using LPDS as an enzyme source because of the distribution of CETP and inhibitor in plasma. CETP binds to lipoproteins, especially HDL, but the association may be disrupted by ultracentrifugation. Inhibitor has been purified from HDL and this implies that it too may be associated with lipoproteins. It is not known to what extent inhibitor is removed from lipoproteins during centrifugation, neither is it known whether there is a difference in activity between bound and free forms. Studies have shown that the majority of CETP (80%) [Groener, J.E.M. *et al* (1984)] is removed from HDL during ultracentrifugation; however it is not known whether the proportion removed by ultracentrifugation is affected by the concentration of HDL in the plasma. Attempts to determine this were not successful (data not shown). A way of circumventing this problem would have been to use dilute plasma to measure CETP activity. However a fivefold range of CETP activities was found in the individuals studied here and dilutions of LPDP in the assay ranged from 1 to 1/5. Thus those plasmas which would be diluted a little would have their CETP activities affected by their endogenous lipoproteins far more than those diluted to a greater extent. Therefore it would be necessary to correct for HDL concentrations anyway.

A net transfer assay was also attempted (data not shown) and although transfer activities similar to those reported in the literature were obtained [Ogawa, Y. & Fielding, C.J. (1985)], the assay lacked sufficient sensitivity to detect changes in activity. This was mainly because the actual mass of lipid transferred was very low.

Antibody detection methods were the methods of choice for mass determinations. At the outset of the project there were insufficient data on the inhibitor protein to consider trying to raise antibodies to it. However an attempt was made to raise antibodies to CETP. Antibodies would allow the measurement of protein mass and could be used to determine whether changes in CETP activity were due to changes in the amount of CETP or changes in its activity. Antibodies which inhibit the enzyme as well as bind to it are useful to examine the effects of inhibiting the action of CETP *in vitro* and *in vivo*. It had been planned to study the effect of inhibiting plasma CETP activity in the rabbit if antibodies which inhibit the rabbit enzyme could be obtained. However since similar studies were carried out by two other groups during the course of the thesis it was decided not to pursue this objective.

Two approaches were attempted for making antibodies in the absence of having a completely pure antigen. One approach was to synthesise a peptide corresponding to a part of the CETP molecule which may be immunogenic. This peptide was used to raise polyclonal antibodies in sheep. Antibodies were successfully raised to this antigen and to the carrier molecule, KLH. Unfortunately the antibodies produced did not recognise native or SDS denatured CETP.

The second approach was to make monoclonal antibodies using partially purified CETP to immunise mice. Mouse spleen cells were fused with NS-1 cells in order to produce clones making antibody to CETP. This approach was not completed but a 'clone' which produced antibodies which cross reacted with other proteins was obtained showing reasonable inhibition of CETP activity and good binding to post CM52 CETP in an ELISA. Immunodetection methods using either monoclonal or polyclonal antibodies would include ELISA, RIA and immunoelectrophoresis. Using any of these detection methods it may have been possible to try and work out the relationship between CETP mass and CETP activity in plasma. This may help give some idea as to which value may be the best to measure in particular circumstances. Detection of CETP mass would also have been useful to try and work out the distribution of CETP between bound and free forms and whether this is affected by HDL concentration. In summary, knowledge of CETP mass in conjunction with CETP activity would have made interpretation of results, especially in the drug and population studies, very much easier.

The monoclonal approach to making antibodies was found to be much more successful than using the peptide approach. Making antibodies using a synthetic peptide can work but unfortunately it is unpredictable as to whether one peptide is better than another for raising antibodies. This problem arises since there are no definite criteria on which one can base a decision to select a peptide that will be immunogenic. Our lack of knowledge on what secondary and tertiary structure a protein will take in solution leaves us entirely unable to predict with confidence which parts of the molecule may be immunogenic. The results obtained here, i.e. that antibodies were raised to the peptide but that these antibodies failed to recognise native CETP illustrate the shortcomings of the approach. Antibodies can be raised to the desired antigen but the antigen is obviously not expressed in the same way in solution as when it is incorporated in the CETP molecule.

With the monoclonal approach the difficulty was screening the clones for activity. Two methods were used, inhibition of CETP activity and ELISA. The ELISA was limited in that pure antigen was not available and instead partially purified CETP was used. Any positives in the ELISA were antibodies which did bind to the post CM52 preparation but could be binding to any one of a number of proteins in that preparation, not necessarily CETP. The inhibition of CETP activity should have been more straightforward since an antibody which inhibits CETP should be picked up. This method would not detect any non-neutralising activities. However it was found that false positives occurred in this assay. Supernatants from clones would cause inhibition of CETP activity but would not bind to post CM52 in an ELISA. It is possible that the cells were producing

something which interfered with the CETP assay e.g. affecting the precipitation of the lipoproteins by heparin manganese. Possibly polysaccharides, especially sulphated ones, or proteins which bind sulphated polysaccharides or even metal ions may have been causing the spurious effects. At the final stages only clones which produced an activity which was positive in both the CETP inhibitory assay and the ELISA were progressed. Other problems encountered with the monoclonal approach were the instability of clones and the length of time that some clones took to grow. Some clones were very slow-growing and increased the time it took to establish a clone and grow up enough cells for inocculation for ascites production.

Antibody production would have been much easier had a pure antigen been available. The CETP protein was not purified to homogeneity here. It was found that after a certain stage the enzyme was unstable and lost activity. The enzyme was greatly stabilised by binding to lipid and the protein could be purified almost to homogeneity by binding to lipid emulsions. Unfortunately it was not possible to remove the protein from the lipid and retain activity. This may have been due to the harshness of the delipidation affecting the activity of the enzyme or due to the fact that lipid may be required to remain bound to the CETP in order for it to function. CETP complexed to Intralipid was electrophoresed on an SDS polyacrylamide gel such that the CETP band could be clearly seen. The gel was then blotted onto nitrocellulose and the CETP bound to the nitrocellulose was cut from the membrane. CETP bound to nitrocellulose was then used as an antigen for immunising mice. However in the final event it was found that immunisation with the less pure post CM52 preparation produced a much better antibody response in mice as measured by inhibition of CETP activity. This suggests that as in the case of the peptide denatured CETP may not be a suitable antigen for raising antibodies capable of inhibiting CETP activity. Had pure antigen been available in quantity screening of clones by ELISA would have been appropriate and it may have been possible to purify antibodies on a CETP column. Also polyclonal antibodies could have been raised by immunisation of sheep with pure antigen.

Rabbit CETP behaved differently from human CETP during purification. Two CETP activities were separated on the phenyl Sepharose column, one eluting with low salt and one eluting with water. These activities appeared to chromatograph differently on the CM52 column when run separately. When the activity peaks from the CM52 peaks were pooled and rerun on a CM52 column then two activities were separated. The result was however inconclusive since one of the peaks only included one measurement of CETP activity. It does appear that rabbit CETP activity differs from human CETP. It is possible that the two peaks of activity seen for the rabbit on the phenyl Sepharose column were an artefact due to overloading of the phenyl Sepharose column. However the activities were still different on a CM52 column. Possibly the rabbit has two plasma CETP activities or plasma CETP can exist in different forms in plasma e.g. free monomeric CETP and aggregated CETP.

Further avenues of study related to CETP purification and antibody production would involve firstly trying to progress the promising clone which demonstrated an anti-human CETP activity. If one were found then this could be used a) to determine the distribution of CETP in plasma to decide whether it is a correct representation to measure CETP activity in the LPDP fraction and b) to investigate the relationship between CETP mass and CETP activity in the plasma. It may also be worthwhile to use the monoclonal antibody to try and purify CETP to homogeneity by immunoaffinity chromatography. Pure CETP would be useful for a standard in measurements of mass and activity and also may be used to raise further antibodies which may allow improvement of the detection techniques. For example a sandwich ELISA using two CETP antibodies may be better than using a one antibody ELISA. Radio immunoassay of CETP may also be a possibility. If polyclonal antibodies are raised then alternative techniques are possible e.g. immunoelectrophoresis.

It would also be interesting to follow up the observation that there may be two rabbit CETP activities. It is necessary to confirm the observation first and then if confirmed, to progress to attempting to purify both activities to homogeneity and sequence them.

2 Studies in Animals

Using the exchange assay a number of studies were carried out to investigate CETP activity in various animal species; rat, rabbit, and man; and the influence of various drugs on this activity. The animal species have been used as models of human lipoprotein metabolism. The rat is often used because of its convenient size, however this species has a different lipoprotein profile from the human with most cholesterol being distributed in the HDL fraction rather than in the LDL fraction as in man. The rabbit is a useful species, however in order to observe hypercholesterolaemia so that lipid lowering effects can be demonstrated, these animals have to be fed a high fat or cholesterol diet or else have to be genetically hypercholesterolaemic. The marmoset is a primate and shows plasma cholesterol levels similar to that found in man without special feeding. Their lipoprotein distribution is not quite so similar to man since a higher proportion of cholesterol is carried in the HDL fraction. Lipid lowering effects have been demonstrated in this animal.

Rats have very low plasma CETP activity and this may in part be responsible for the lipoprotein distribution in this species. High HDL cholesterol levels and low LDL cholesterol levels would result from negligible CETP. This would suggest that reverse cholesterol transport is carried out by the HDL particles in this species since cholesterol cannot be transferred to lower density particles to allow the cholesterol to be taken up by LDL receptors. There is some evidence for the role of HDL in delivering cholesterol to the liver in the rat. However there is mounting evidence that cholesterol esterification can take place in LDL and it is possible that this process occurs in the rat. An alternative hypothesis is that CETP activity could be expressed at certain times and may in fact show a circadian rhythm as do some other enzymes of cholesterol metabolism in the rat.

In fact it has been demonstrated that rat plasma does contain CETP activity but that this activity is normally suppressed by the presence of CETP inhibitor protein. In order to test for the presence of a circadian rhythm for CETP, plasma CETP activity was measured in two groups of rats, one group in the mid-light cycle and the other was in the mid-dark cycle, a time at which another enzyme HMGCoA reductase is at the peak of its circadian rhythm. All the plasma CETP activities measured were very low and although a significant difference between the activity of the enzyme mid-light cycle and mid-dark cycle was noted at six weeks after adaptation to the light/dark cycle, the difference was only a very small fraction of what one might expect to see if inhibitor was removed. Thus it was concluded that there was no circadian rhythm of CETP activity in these rats or if so, the peak of activity was so sharp that it could not be detected at twelve hour intervals.

The studies in the rabbit first of all confirmed that fat feeding normal Dutch Belted rabbits resulted in hypercholesterolaemia accompanied by an increase in plasma CETP levels. It was also found that in a breed of rabbits (Froxfield) which shows a heritable hyperlipidaemia due to an LDL receptor defect demonstrated an increase in plasma CETP levels similar to that seen in the Watanabe rabbit.

It is not known whether this rise in plasma CETP activity is an independent response or whether it is secondary to a rise in plasma total cholesterol or a rise in cholesterol in a particular lipoprotein subfraction. Drug studies are a means to test whether it is possible to alter cholesterol levels without altering CETP activity or *vice versa.*

Three classes of drug were looked at in the Froxfield rabbit model of hypercholesterolaemia; an ACAT inhibitor, a β_2 agonist and two antioxidants. The β_2 agonist, thought to be able to reduce the amount of cholesterol stored in a cell by stimulating neutral cholesteryl ester hydrolase (NCEH), had no effect on either plasma total cholesterol or plasma CETP activity. The ACAT inhibitor did not significantly change plasma total cholesterol but did significantly increase plasma HDL levels. This increase in HDL levels was not accompanied by a change in CETP activity. Since a negative correlation between plasma HDL levels and CETP activity has been demonstrated one might expect changes in the HDL fraction to be accompanied by changes in CETP activity, more so than to changes in total cholesterol which shows no such correlation. This was not found to be the case in this experiment where HDL levels changed but total cholesterol did not. However this study was inconclusive in that the measurement of plasma HDL levels was not entirely satisfactory.

Probucol is an interesting drug in that despite causing a decrease in HDL cholesterol, FH patients treated with the drug show xanthoma regression. The regression is correlated with the decrease in HDL. Watanabe rabbits treated with probucol show reduced arterial lesion formation. In this study where rabbits were fed probucol for 12 weeks no change in either plasma total cholesterol or CETP activity was found. It was expected that a fall in total cholesterol would be seen and plasma measurements of probucol demonstrated that the drug was being absorbed. There was evidence that arterial lesion progression

was reduced in the treated animals. Thus in the absence of any effect on CETP activity an effect on the lesions was seen. This tends to negate the idea that a mechanism involving CETP is responsible for the reduction in lesion formation.

Cases have been observed in humans where plasma CETP activity has been affected by treatment with probucol. Two familial hyperalphalipoproteinaemic (FHALP) patients, one with impaired CETP activity and both showing an abnormal direction of CE net transfer from LDL/VLDL to HDL, had normal CETP activity and direction of net transfer after treatment and a decreased HDL_2 cholesterol. Type II hyperlipidaemic patients treated with probucol had an increased CETP activity and decreased HDL_2 while HDL_3 was unchanged. In the larger study of normal humans on probucol investigated here, despite a pilot result where two volunteers showed an increase in CETP activity, there was no significant effect on CETP activity although both total cholesterol and HDL cholesterol were significantly decreased. It is possible that an effect on CETP activity would only be seen if CETP activity was impaired to start with i.e. probucol 'normalises' CETP activity. A possible mechanism for this could be via the CETP inhibitor protein. If impaired CETP activity were due to increased inhibitor protein, then decreasing the amount of inhibitor would normalise the activity. It is interesting that the post probucol samples appeared to contain less inhibitor than the control samples. Thus in these normal samples perhaps inhibitor is being affected but this is not apparent as would be in diseased samples where CETP activity was compromised.

Probucol is an antioxidant but it is not known whether its effects on slowing the progress of atherosclerotic lesions is due to this property. The effect of another unrelated antioxidant was tested for its effect on CETP activity in rabbits. It was found that GR44966X decreased plasma CETP activity, the opposite of what might be expected for probucol. It is not known how this drug affected plasma cholesterol levels. Before this result can be followed up it should be confirmed in a larger group of animals and its effect on plasma cholesterol monitored.

HMGCoA reductase inhibitors were used to treat both marmosets and a group of human hyperlipidaemic patients in the clinic. Significant decreases in plasma cholesterol were seen in both species but differing effects on plasma CETP were seen. In the marmosets there was no effect of treatment on CETP activity. However in the humans, CETP activity was reduced over the same time period as the total cholesterol. Again it is possible that changes in CETP are seen only when the plasma levels are abnormal to start off with. The marmosets used here were normal but the human patients had high plasma cholesterol and high CETP activities which were decreased towards more normal levels by drug treatment. A second difference here is the time scale over which the experiments took place. The marmosets were treated for only one week whereas the humans were treated for up to six months. However changes in plasma cholesterol were seen in the 7 day experiment in marmosets. It is possible that CETP activity changes over a longer period of time. These differences may also be just reflecting species differences.

No clear pattern of behaviour for CETP emerges from these experiments. They do point to conditions which may have importance when looking at changes in CETP activity. Species may respond differently depending on the importance of CETP inhibitor in affecting overall CETP activity. It seems that in the rat there is a greater expression of inhibitor than there is in the rabbit. These experiments and others also seem to indicate that drugs may have more of an effect on CETP levels when activity is impaired or raised since it is on these occasions that changes in CETP activity are demonstrated. The length of time over which dosage takes place may also be an important factor. Although long term experiments seem to show that changes in CETP activity mirror changes in total cholesterol e.g. in cholesterol fed rabbits [Quig, D.W. & Zilversmit, D.B. (b) (1988)] or as in the case here of treating hyperlipidaemics with Simvastatin, an effect was not seen in the short term experiment using marmosets.

It is not possible from these experiments to sort out a cause and effect relationship between CETP and total cholesterol. It is not known whether the level of these parameters are controlled independently or whether one follows a change in the other. If a drug treatment may be found which affects each parameter separately then it may be possible to get some insight into this question. Inhibition of CETP activity may not be directly due to inhibition of the enzyme. It may result from changes in inhibitor concentration or changes in CETP distribution between HDL particles and the plasma.

The question arises as to whether it would be therapeutically beneficial to inhibit CETP. Certainly plasma levels are raised in hyperlipidaemics; and in FHALP (a condition predisposing to high HDL levels and longevity) plasma CETP levels are low or reduced. However in experiments where CETP has been inhibited *in vivo* by antibodies [Whitlock, M.E. *et al* (1989)], HDL CE rose in the absence of an increase in LCAT activity and the clearance of ^{3}H-cholesteryl ether from both the HDL and the plasma compartments was decreased. Thus although HDL cholesterol rose it appeared that HDL CE catabolism was decreased. Therefore the usefulness of a CETP inhibitor is still unclear.

3 Secretion of CETP by Cells

J774 cells, macrophages, HepG2 cells and CaCo2 cells were studied for control of CETP secretion. These cells have been reported to secrete CETP activity. It was confirmed in the study carried out here that these cells do secrete CETP. However the amount of CETP secreted was very low and it appeared that the assay system used here was not sensitive enough to detect accurately the amount of activity found in the supernatants. Measures to increase the sensitivity of the assay; incubation for a longer time and concentration of cell media did improve detection, but transfer measured was still not much more than 5% transfer over the course of the assay, a level of detection which was near the limit of sensitivity for the assay. Thus it was found that measurements of CETP secretion by cells and its regulation by effectors was not reproducible and changes in amount of CETP activity were not large compared to the error on the CETP measurement itself.

This inability to detect changes in CETP activity may be the reason why it was difficult to reproduce results quoted in the literature e.g. stimulation of CETP secretion in CaCo2 cells by free fatty acids. The level of CETP secretion detected here appears to be lower than that quoted in the literature but not grossly so. It is possible that the cells used here were suffering from an undetected infection (e.g. mycoplasma) or were not at an appropriate passage number, being either too high or too low. It is possible that at a high passage number the cell line loses characteristics of the original parent cell. Cell viability in serum-free medium becomes a problem after 24 hours and it is also possible that some cells may be producing CETP inhibitor protein as well as CETP thus making it appear that no CETP is secreted. Media from some experiments were passed over a phenyl Sepharose column but this did not reveal any latent CETP activity so this possibility may be unlikely. The macrophages on occasion did show quite large amounts of secretion (>10% transfer/60μl medium) but this varied greatly from donor to donor and is not reproducible for the same donor either. Macrophages are a cell type known to demonstrate great variabilty between donors. There were even great differences in cell yield between different donors. Since the results from the cell secretion studies were not reliable it is impossible to draw any conclusions as to the effects of various treatments on the cells.

Thus alternative ways were considered for measuring the effect of various treatments on CETP levels. It is possible to look at the level of mRNA which indicates at least the rate of transcription if not the rate of translation of CETP. It was found that CETP mRNA could be detected by slot blotting and hybridisation to an oligonucleotide probe. Using 50μg of total RNA, CETP message could be detected in macrophages, J774 and HepG2 cells. Unfortunately the hybridisation of probe to the membrane did not appear to be quantitative. This may have been due to unequal loading of RNA onto the membrane or due to the fact that the hybridising probe found RNA on the top of a loaded area more accessible than that underneath or may find some parts of the membrane more accessible than others due to positioning of the filter in the hybridising bag. It was clear from an experiment where control and PMA treated cells were compared for hybridisation of their RNA to a CETP probe that it was difficult to determine quantitative differences between them.

Another technique which was started was a nuclease protection assay where hybridisation occurs in solution avoiding the problems of accessibility of RNA on a filter. It was first necessary to validate the hybridisation techniques to test that CETP mRNA could be detected on a Northern blot at the correct position, to test that the probe hybridised correctly to the CETP mRNA. Running both total and purified mRNA from various cell types on a formaldehyde gel and blotting onto nitrocellulose, it was found that CETP probes did not give a signal when hybridising to the membrane blots. Using an actin probe as a control it was discovered that this was due to two factors. Firstly, the CETP message may be present in smaller amounts than the actin message and so is more difficult to detect. Secondly, degradation of RNA, which was not detectable by eye with ethidium bromide staining was occuring since the actin probe bound to a

smear of low molecular weight material. It is possible that RNA is being degraded by RNAases introduced either at the purification stage or at the sample preparation and gel running stage.

To improve upon these results the procedures for handling RNA would have to be stringently assessed and altered so that as little contamination of the RNA samples with degrading enzymes as possible took place. It is possible that the partial alkaline hydrolysis of the RNA gels before blotting was too harsh and may have been degrading the RNA at that stage. This would account for a more diffuse arrangement of a particular mRNA but would probably not be responsible for the low molecular weight RNA because in order for the RNA to reach this position on the gel it would have had to have been degraded before electrophoresis.

To test whether little signal was being obtained due to low concentration of CETP message, it would be possible to amplify the message using polymerase chain reaction. Preliminary experiments showed that a sequence within the CETP gene could be amplified using combinations of 4 chosen primers, two sense and two antisense. The PCR should be able to amplify sequences from both genomic DNA and mRNA after reverse transcription to cDNA. Thus this technique may be employed to detect whether any CETP message is present. It is not easy to use this technique in a quantitative manner at present.

Once the problems with RNA degradation have been sorted out and one is satisfied that CETP mRNA is present, one can return to testing whether a CETP probe can hybridise to CETP mRNA on a Northern blot. The nuclease protection assay may be used to measure the amount of mRNA in a particular sample though it is possible that a slightly longer probe (100 to 200 base pairs) would be required for best effects. A nuclease protection assay when set up would be very sensitive and could be used to look for changes in CETP secretion brought about by various effectors. Effectors of interest would include cytokines e.g. IL-1 and TGFβ and also agents which could alter the cholesterol balance of the cell. It would be interesting to see whether a cell would respond to changes in its cholesterol levels by altering output of an enzyme which may be involved in flux of cholesterol in and out of the cell.

4 CETP RFLP Analysis in a Population

The RFLP analysis in the population of high and low HDL individuals produced some interesting results. Firstly a significant relationship between an MspI polymorphism of the apoAI gene and plasma LCAT activity was revealed. Individuals with the M_1M_2 genotype had significantly higher LCAT activities ($P<0.004$) than those with genotype M_1M_1. It is not clear how variation in the apoAI gene would affect plasma LCAT activity. It may be a result of the method of preparation of LCAT for the analysis. Since LPDP is used as the enzyme source then it is possible that endogenous apoAI which has come free of the HDL during ultracentrifugation may be playing a part in activating the enzyme and variation is being expressed via this mechanism. However it does seem

unlikely that this effect would be seen in the presence of a large amount of exogenous apoAI. It is possible that the apoAI polymorphism may be marking for another area on the chromosone which may somehow affect LCAT activity.

The main aim of the study was to investigate whether variation at the CETP gene influenced plasma CETP activity and HDL levels. It was found that individuals with the TaqIB rare allele, B_2, had significantly lower CETP activity ($P<0.01$). This result is not surprising since it might be expected that variation in the structure of the CETP gene detected directly, or more likely indirectly by linkage, by the TaqIB polymorphism can affect plasma enzyme levels. What is more interesting is that this same polymorphism is seen to be associated with HDL levels. There is a significantly ($P<0.002$) different distribution of individuals between the genotypes B_1B_1, B_1B_2 and B_2B_2 in the high and low HDL groups.

It has previously been suggested that there is a negative correlation between HDL levels and CETP activity both from measurements of both parameters within a population and from the example of FHALP people who have no or negligible CETP activity. It is impossible to sort out from this evidence alone a cause and effect relationship. The evidence from the RFLP study implies that CETP may have an effect on HDL levels which supports the idea that CETP may have an antiatherosclerotic role. It has recently been reported [Brown, M.L. *et al* (1989)] that one FHALP family displays a splicing defect in CETP pre mRNA resulting in an inherited deficiency of CETP. This demonstrates that a primary defect in CETP results in a phenotype demonstrating high plasma HDL levels.

One must be careful not to draw too many conclusions from RFLP analysis. For a start what is being measured is not necessarily the mutation in the gene which is responsible for the variability itself but more likely a site which cosegregates with the mutation of interest. Since there is a distance between these two points there is a possibility of recombination between them so that there is not a 100% correlation between the presence of the RFLP site and the site responsible for the variation in CETP activity. It is not even necessarily true that the site responsible for differences in activity is in the CETP gene itself. It may be in a nearby related gene. For example it is feasible that if the gene for CETP inhibitor protein were on chromosone 16 the RFLP in the CETP gene could mark for a mutation in the CETP inhibitor gene which could also influence plasma CETP activity. If measurement of CETP mass could be carried out then this could sort out whether the differences in CETP activity associated with the RFLP alleles were due to changes in CETP mass or were due to changes in factors which influence plasma CETP activity.

Major reservations with the RFLP study carried out here were small population size, the unequal distribution between males and females especially since they were difficult to analyse separately due to small numbers, and the number of tests carried out. Since a large number of significance tests were carried out here testing five RFLPs against four plasma parameters (20 tests) then it is possible that by chance alone some

of these tests will prove significant. A crude way of accounting for this is to multiply the significance level obtained by the number of tests carried out. If this is done then the significance of the association between the TaqIB RFLP and plasma CETP activity is lost but that with the plasma HDL levels remains. It is also very important to determine whether the proportion of plasma CETP activity found in the LPDP is affected by the levels of plasma HDL as discussed earlier. If this were the case then this may account partly for the results seen in the RFLP analysis.

This work confirms the findings of Kondo *et al* [Kondo, I. *et al* (1989)]. They found an association between the TaqIB polymorphism and apoAI levels and this effect was not seen in smokers when the population was divided into smokers and nonsmokers. The work here demonstrates a similar effect in a different population and and suggests a mechanistic link between a CETP RFLP, low CETP activity and high HDL levels. There was no information on our population to allow us to look at the effect of smoking.

It is obviously important to try and repeat this result with an improved study design. Improvements would include increasing the population size to at least 200 with 50% male and 50% female individuals. If possible CETP mass as well as CETP activity should be measured.

5 Summary

The aims laid out at the beginning of this project were:-

1) To assess the relationship between plasma CETP activity and CETP mass.

2) To inhibit CETP activity *in vivo*.

3) To examine the effects of drugs affecting cholesterol metabolism on CETP activity.

4) To identify factors controlling CETP secretion by cells in culture.

5) To test for any relationship between RFLPs of the CETP gene and plasma CETP activity and HDL levels.

An assay was set up which could measure CETP activity in plasma, but an antibody to CETP which may have been used to assay for CETP mass was not produced. Thus the relationship between these two parameters could not be assessed. A good monoclonal antibody has been produced by another group [Yen, F.T. *et al* (1989)] and information on this relationship may soon be forthcoming. It would be interesting to find out why some animals have such an excess of CETP activity. When CETP activity was inhibited by antibodies in the rabbit, the plasma CETP levels only needed to return to 20-30% of the level normally found in plasma for any effects on the lipoprotein caused by antibody inhibition to be normalised. Also when inhibitor is removed from pig plasma using a phenyl Sepharose column, much more inhibitory activity could be eluted than was required to inhibit the pig transfer activity separated. It might be expected that if CETP activity and CETP inhibitor protein are present in such large excesses then control of activity by altering the amounts of these proteins might be difficult. However some

animals obviously do exhibit a tight control on CETP activity and their plasma levels are completely inhibited. Possibly there are further areas of control in the plasma compartment e.g. binding and dissociation of CETP to its substrate lipoproteins. It has been demonstrated that binding of CETP to HDL is increased by conditions which increase CETP activity e.g. in the fatty acid stimulation of CETP activity. It may be more appropriate to examine what is happening at the local level in the lipid transfer complex. Local control systems of binding of CETP or inhibitor to lipoproteins may be obscured by measurements of excesses of free CETP and inhibitor. Now that more information on the CETP inhibitor is available direct mass measurements of inhibitor may also be possible in future.

Since studies on the inhibition of CETP *in vivo* in the rabbit by both polyclonal and monoclonal antibodies were published in 1989 the search for a rabbit monoclonal antibody was discontinued. The results from these published studies were interesting. HDL CE levels were increased as would be expected but there was no increase in LCAT activity. Thus this increase in CE was probably due to decreased catabolism. Supporting this it was found that the clearance of labelled cholesteryl ether from the HDL and plasma compartments was delayed and there was decreased uptake of label into the liver. Conversely when CETP was injected into rats labelled apoAI containing HDL particles were cleared from the blood more quickly and there was an increase in accumulation of CE in the liver. These experiments indicate that CETP is necessary for RCT and CE may be being delivered to the liver for excretion by the lower density lipoproteins, LDL and VLDL, after transfer from HDL. Since LCAT activity is not affected by CETP antibodies it seems that CETP is not involved in cholesterol removal from the cell unless it interacts with the cell directly. It is difficult to accommodate the evidence of increased CETP activity in hyperlipidaemia and low or zero CETP activity in FHALP people with a role for CETP in RCT when hyperlipidaemia is associated with increased risk of atherosclerosis and FHALP is associated with longevity especially as in the latter case the lack of CETP is a primary defect. These two conditions have led to proposals that CETP interferes with the RCT pathway by diverting cholesterol away from HDL which is returning CE to the liver. Several groups have discovered a negative correlation between CETP activity and HDL levels. Perhaps the paradox here can be explained by the time scale over which the *in vivo* antibody inhibition studies took place. CETP was inhibited for only at most 48 hours. It may take longer than that for a new steady state RCT pathway to be reached, and what has been measured in these studies may only be a transitional state. It is certainly true that in the FHALP family, which has a genetic error leading to improper splicing of the CETP gene, affected individuals are not dying of premature heart disease. Another interesting result that arose from the inhibition of CETP by antibodies is that CE still appears in LDL in the absence of any measurable CETP activity. It appears then that LDL can act as a substrate for LCAT. Perhaps animals lacking CETP activty employ this mechanism for getting CE into LDL and to the liver via the LDL pathway.

The effect of several drugs on CETP activity in several species was studied. No consistent effects were observed. Certainly hyperlipidaemia due to a genetic fault and to fat feeding led to increased CETP levels as has been reported previously. Treatments which decreased CETP activity were GR44966X, an antioxidant, in Froxfield rabbits and Simvastatin in hyperlipidaemic humans. The effect of an HMGCoA reductase inhibitor in another species, the marmoset, did not agree with the finding in humans. There may be two reasons for this. One is that the time scales of the experiment were different and the other is that the marmosets had normal cholesterol levels whereas the humans had abnormally high cholesterol levels. It seems to be a recurring theme that CETP levels can revert to normal levels once the individual is treated. Increased CETP is lowered by Simvastatin or omega-3-fatty acids and impaired CETP activity is increased by probucol in some cases. It is possible that control of CETP activity only becomes critical once the lipoprotein metabolism has gone awry. Changes in CETP activity may only be reflecting abnormal cholesterol levels or lipoprotein compositions rather than causing them. Treatment with drugs may be affecting CETP activity because they are normalising cholesterol and lipoprotein metabolism in other ways e.g. HMGCoA reductase inhibitors will decrease cholesterol biosynthesis and reduce plasma cholesterol. Certainly when one can see decreases in CETP activity mirroring decreases in plasma cholesterol in the Simvastatin treated patients (where CETP is abnormally high), when changes in CETP activity in the marmoset are not seen although plasma cholesterol levels are reduced, it does imply that the changes in CETP activity are secondary to the fall in plasma cholesterol rather than the other way around.

Studies into the control of CETP secretion were frustrated by technical difficulties. It would be interesting to know whether increases in plasma CETP activity are due solely to changes in enzyme activation or whether there is increased mass. Obviously if there is increased mass then this makes control of the synthesis of CETP very important. The experiments where vectorial secretion of CETP by CaCo2 cells can be increased by free fatty acids imply that at least in some cases, as in the post-prandial state, CETP synthesis is upregulated. The main source of CETP in the body is not known. It has been calculated from the rate of secretion in HepG2 cells that the liver could account for the entire body synthesis of CETP. Liver does secrete CETP but this may be contributed to by the Kupffer cells as well as the hepatocytes and in fact little CETP mRNA synthesis has been detected in HepG2 cells. Certainly the effect of free fatty acids on CaCo2 cells cannot be ignored. Possibly this control may be of local importance involved mainly with the processing of lipids after a meal.

The study of the relationship between RFLPs of the CETP gene, plasma CETP levels and plasma HDL levels is interesting because it suggests that CETP may have a direct effect on HDL levels and that CETP is not passively reflecting plasma cholesterol levels. This evidence must be treated cautiously however as it is not directly measuring the genetic mutation responsible for the effect but is just following a marker for it and so relies upon statistical interpretation. Because this is so, the study ought to be repeated

in a larger population so that the statistical power of analysis can be strengthened. It is also important to follow up the genetic study with a study that can investigate the metabolic mechanism that results.

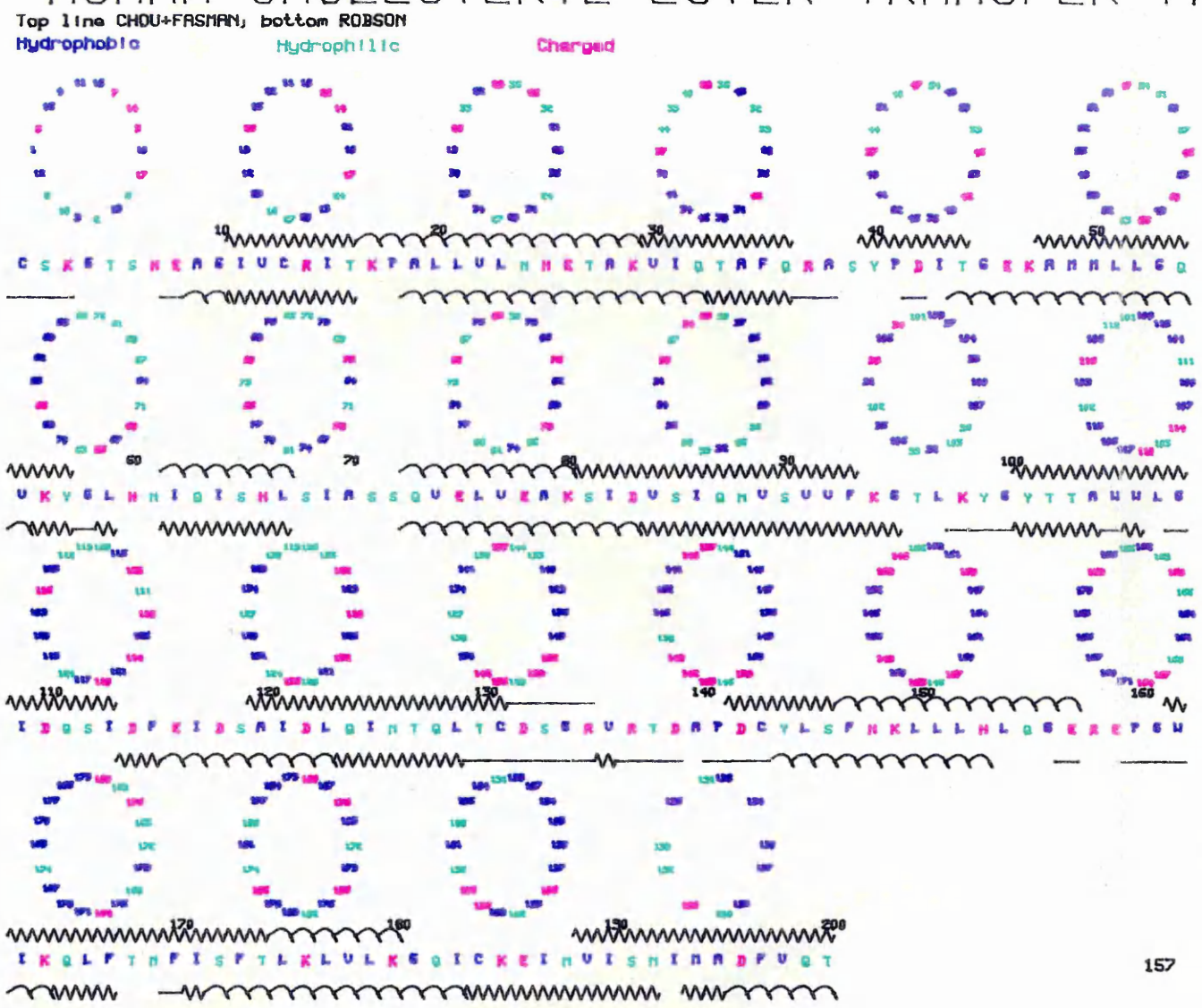
What roles does CETP play in cholesterol metabolism? The answer is still unknown but it does seem increasingly likely that the role is an important one as more mechanisms for the regulation of CETP activity are discovered. It also seems likely that the role is a varied one as evidence is strong for its involvement in a number of areas especially reverse cholesterol transport and post-prandial processing of lipids.

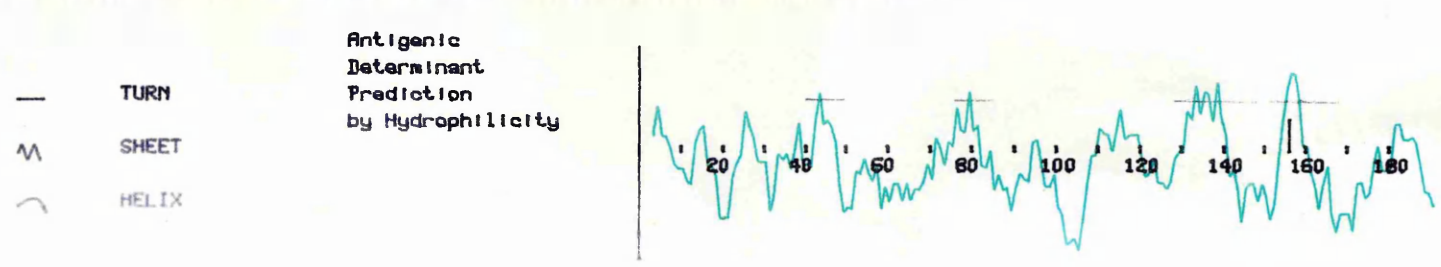
APPENDIX I

HUMAN CETP PREDICTED SECONDARY STRUCTURE FROM THE DEDUCED AMINO-ACID SEQUENCE

Using the published cDNA sequence and predicted protein sequence [Drayna, D. *et al* (1987)] the 'Predict' computer programme (Peter Murray-Rust, Glaxo Group Research Ltd, Greenford) was used to carry out Chou & Fasman and Robson predictions for secondary structure. A hydrophilicity/hydrophobicity plot is also shown. Hydrophilic residues are plotted above the line and hydrophobic residues below the line.

HUMAN CHOLESTERYL ESTER TRANSFER PROTEIN NATURE 18JUNE1987 P633





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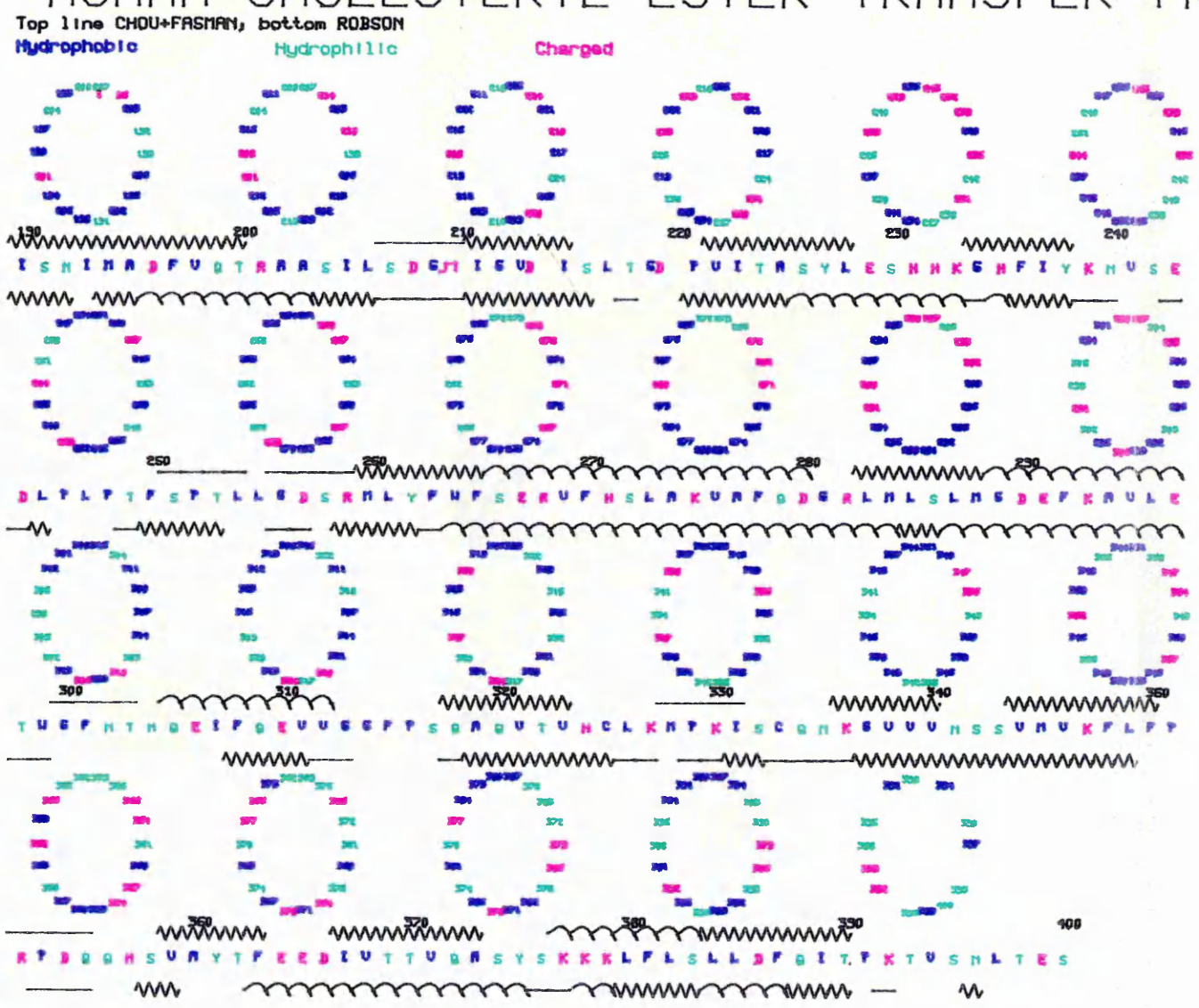

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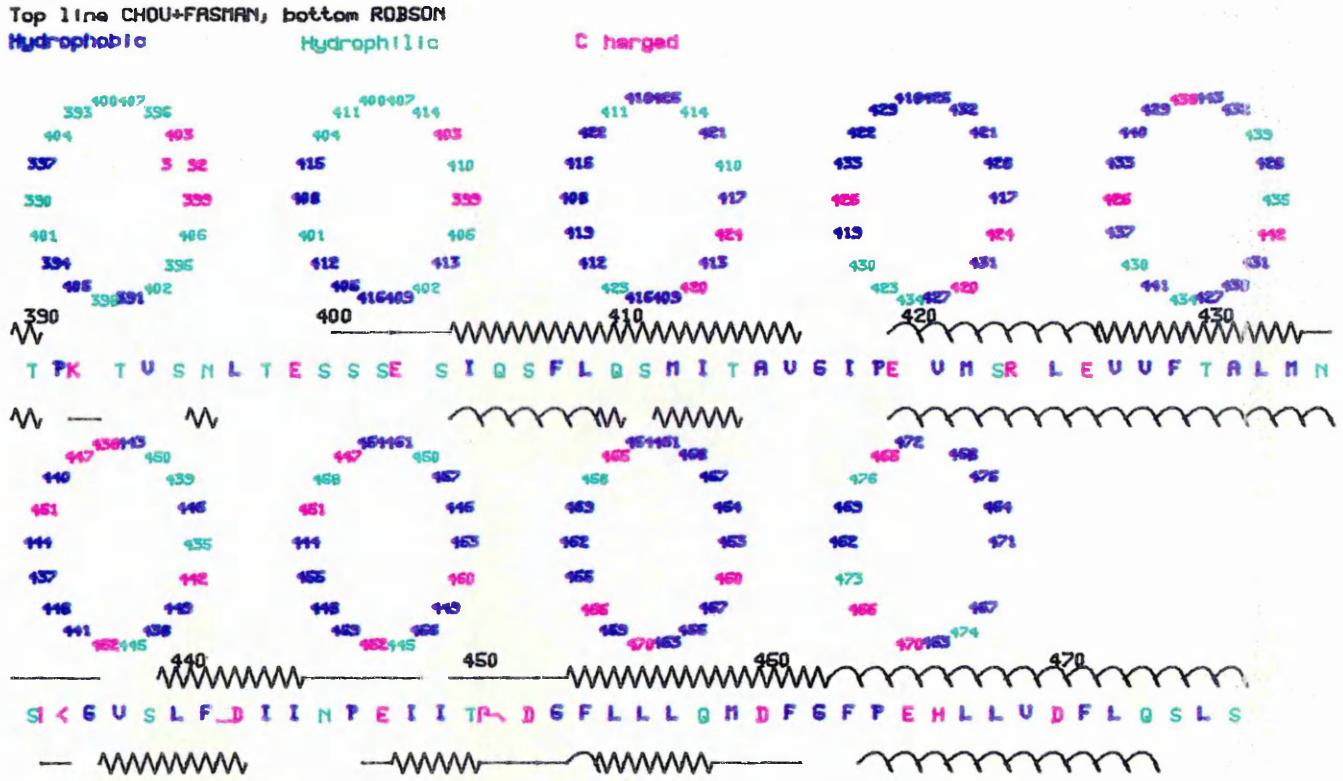

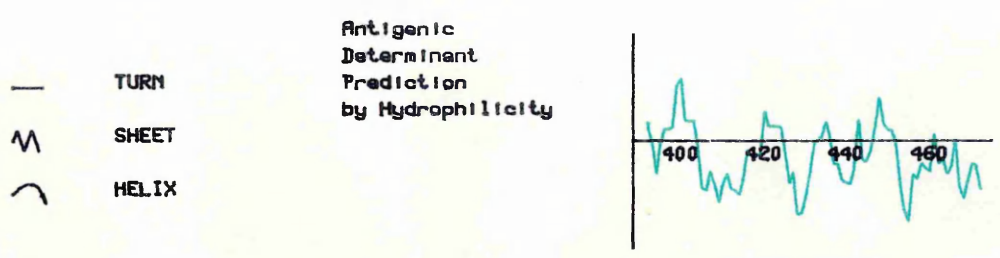

APPENDIX 2

CALCULATION OF THEORETICAL MELTING TEMPERATURE OF DNA

$Tm = 16.6 \log[M] + 0.41 [P_{GC}] + 81.5 - P_M - B/L - 0.65\ Pf$

M = molarity of Na (_0.75M)

P_{GC} = % G or C in single strand (between 30-60%)

P_M = % mismatched base (should be 0)

B = 675

L = probe length in bases (75)

f = percentage formamide (50%)

Stringency is taken as being 12°C below the melting temperature

$Tm = 16.6 \log 0.75 + 0.41 \times 31 + 81.5 - 0 - (675/75) - 0.65 \times 50$

$Tm = -2.07 + 12.71 + 81.5 - 9 - 32.5$

$Tm = 50.6°C$

Stringency = 38.6°C

APPENDIX 3 - ABBREVIATIONS

ACAT	acyl CoA cholesterol acyl transferase
apo	apolipoprotein
BSA	bovine serum albumin
CE	cholesteryl ester
CETA	cholesteryl ester transfer activity
CETP	cholesteryl ester transfer protein
CM	chylomicron
CO	cholesteryl oleate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
FFA	free fatty acid
FH	familial hypercholesterolaemia
FHALP	familial hyperalphalipoproteinaemia
HDL	high density lipoprotein
HSA	human serum albumin
IL-1	interleukin-1
KLH	keyhole limpet haemocyanin
LCAT	lecithin cholesterol acyl transferase
LDL	low density lipoprotein

LP	lipoprotein
LPDP	lipoprotein depleted plasma
LPDS	lipoprotein depleted serum
LPS	lipopolysaccharide
LTP	lipid transfer protein
MOPS	morpholinopropanesulphonic acid
mRNA	messenger RNA
NCEH	neutral cholesteryl ester hydrolase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
pCMPS	parachloromercuriphenylsulphonate
PL	phospholipid
PMA	phorbol myristate acetate
RCT	reverse cholesterol transport
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	sodium chloride, sodium citrate buffer
TBS	Tris buffered saline
TEMED	N,N,N',N',-tetramethylethylenediamine
TG	triglyceride
TMB	tetramethyl benzidine
TO	triolein
Tween 20	polyoxyethylenesorbitan monolaurate

VLDL very low density lipoprotein

APPENDIX 4 - SUPPLIERS OF REAGENTS

Alpha Laboratories 40 Parham Drive Eastleigh Hampshire U.K.	Wako kits for FFA determinations
Amersham International plc White Lion Road Amersham Bucks U.K.	Radiochemicals DNA Multiprime labelling kit Hybond N
Amicon Ltd Upper Mill Stonehouse Glos U.K.	Apparatus and membranes for ultrafiltration
Baker Instruments Shirloy Avenue Windsor Bucks U.K.	Triglyceride assay kit
BDH Ltd Broom Road Poole Dorset U.K.	General chemicals

Beckman-RIIC Ltd Progress Road Sands Industrial Estate High Wycombe Bucks U.K.	Centrifuges, rotors and tubes
Bio-rad Laboratories 3rd & Regatta Boulevard Richmond CA 94806 USA	Protein assay Gel matrices
Boehringer Mannheim Gmbh Bell Lane Lewes East Sussex U.K.	Kits for cholesteryl ester and triglyceride Reagents for fluorescent CE assay
British Biotechnology Ltd Watlington Road Cowley Oxford U.K.	Primers for PCR
Cambio 34 Millington Road Cambridge U.K.	Taq polymerase
Costar Corporation 205 Broadway Cambridge MA USA	Transwells

CRB Button End Harsten Cambridge U.K.	KLH
Denley Instruments Ltd Natts Lane Billinghurst Sussex U.K.	Spiralmix
Difco Laboratories Detroit Michigan USA	Freunds adjuvant
Dynatech Laboratories Daux Road Billingshurst West Sussex U.K.	Microtitre plates
Evans Medical Horsham Surrey U.K.	Streptomycin
Gibco PO Box 35 Trident House Renfrew Road Paisley U.K.	Tissue culture medium including Biocult Foetal calf serum Non essential aminoacids Molecular biology reagents Restriction enzymes

Jones Chromatography Tir-Y Bgrta Industrial Estate New Road Hengoed Mid Glamorgan U.K.	Spherisorb
Kabivitrum Ltd Riverside Way Uxbridge Middx U.K.	Intralipid
Kodak Laboratory and Research Products Acornfield Road Knowsley Industrial Park North Liverpool U.K.	Film developing solutions Cab-O-Sil
Labmedic Ltd Maxron NSE Green Lane Romiley Stockport Cheshire U.K.	Sebia lipofilm kits
Lancaster Synthesis Ltd Eastgate White Lane Morecambe Lancs U.K.	Probucol

MSE Manor Royal Crawley Sussex U.K.	Sonicator
National Diagnostics Unit 3 Chamberlain Road Aylesbury Bucks U.K.	Ecoscint
A/S NUNC Kamstrupvej 90 Kamstrup DK-4000 Roskilde Denmark	Tissue culture plastics ELISA plates
Oncor Inc Box 870 Gaithersburg MD 20877 USA	Actin probe Hybridisation fluid
Packard Instruments Co. Ltd 2200 Warrenville Road Downers Grove Illinois USA	Scintillation fluid
Peptide and Protein Research University of Reading Reading U.K.	Synthetic peptide

Perkin Elmer Ltd Post Office Lane Beaconsfield Bucks U.K.	Luminescence spectrometer
Pharmacia LKB Biotechnology Midsummer Boulevard Central Milton Keynes Bucks U.K.	Gel matrices Molecular biology reagents Polybuffer 94 Ficoll Paque dNTP mRNA purification kit Nitrocellulose
Sigma Chemical Co. Ltd Fancy Road Poole Dorset U.K.	General chemicals Antibodies Hydroxylapatite Con A Sepharose Human serum albumin Pristane Poly-D-lysine
Special Diet Services Ltd (SDS) PO Box 705 Witham Essex U.K.	Animal diets
Vestric Ltd Northolt Middx U.K.	Penicillin
Whatman Ltd Springfield Mill	Gel matrices

Maidstone

Kent

U.K.

APPENDIX 5 - REFERENCES

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