STUDIES ON THE INTERACTION OF RAT SERUM PHOSPHORYLCHOLINE BINDING PROTEIN (PCBP) WITH PLASMA LIPOPROTEINS

UDAY SAXENA

STUDIES ON THE INTERACTION OF RAT SERUM PHOSPHORYLCHOLINE BINDING PROTEIN (PCBP) WITH PLASMA LIPOPROTEINS

BY

© UDAY SAXENA B.Sc. M.Sc.

A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Department of Biochemistry
Memorial University of Newfoundland
St. John's, Newfoundland

September, 1986

ISBN 0-315-43372-8

ABSTRACT

Rat serum phosphorylcholine binding protein (PCBP) was previously shown to inhibit heparin-lipoprotein-precipitation reaction. Comparison of the effect of PCBP on heparin-lipoprotein precipitation reaction with similar proteins from other species revealed a striking difference between the glycosylated rat PCBP and female hamster FP and the non-glycosylated varieties (human and rabbit CRP's). Whereas FP shared the inhibitory effect with rat PCBP, human and rabbit CRP failed to inhibit the precipitation reaction suggesting a role of the sialic acid residues on PCBP and FP in the mechanism of inhibition of heparin-lipoprotein precipitation reaction.

The binding of PCBP to multilamellar liposomes prepared with egg phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) was studied and the binding was found to be Ca^{2+} dependent and required the incorporation of 25% LPC into the liposomes. The binding was inhibited by phosphorylcholine (P-choline). Substitution of phosphorylcholine head groups by phosphorylethanolamine and phosphorylserine on the PC of the liposomes reduced the binding considerably indicating involvement of the phosphorylcholine head groups in the binding of PCBP to liposomes.

Studies on the binding of human plasma lipoproteins to PCBP immobilized on Sepharose showed that very low density lipoproteins (VLDL) were partially bound and the bound fraction contained higher amounts of apoprotein (apo) B and

E. All the low density lipoproteins (LDL) were bound to the column. In case of high density lipoproteins (HDL) only a small fraction was retained on the column but that bound fraction contained all the apo E and Lp(a) applied. The binding of lipoproteins was Ca^{2+} dependent and the bound lipoproteins were eluted by a P-choline gradient. Prior equilibration of Sepharose-PCBP column with P-choline prevented the binding of LDL but the removal of sialic acid from PCBP had no effect on the binding of LDL to immobilized desialylated PCBP. Chemical modification of arginyl residues on apo B in LDL resulted in marked reduction of binding whereas modification of lysine residue had no effect. The results suggest a perferential binding of apo B and E containing lipoproteins with PCBP.

Investigations to examine the effect of PCBP on the binding of human LDL to LDL receptors on liver membranes from estradiol treated rats showed that PCBP inhibited the binding of LDL to receptors. Preincubation of liver membranes with PCBP did not affect the binding of ^{125}I-LDL to the membranes. Gel filtration analysis of the incubation products from the LDL-receptor assays showed a concentration dependent binding of ^{125}I-PCBP to LDL. These results suggest that the inhibitory effect of PCBP is probably due to fluid phase interactions between LDL and PCBP and not due to the binding of PCBP to the LDL receptor site.

The effectiveness of Sepharose-PCBP columns to bind

plasma VLDL and LDL from control and hypercholestrolemic rabbits when used in a extracorporeal plasmapheretic system was tested. Results showed that Sepharose-PCBP columns bound some circulating plasma lipoproteins and most (>90%) of the bound lipoprotein fraction contained VLDL + LDL. The results obtained in this study support the possibility of a role of rat PCBP and similar circulating phosphorylcholine binding proteins of other species in lipoprotein metabolism.

ACKNOWLEDGEMENTS

I wish to express my deep gratitude towards my supervisor, Dr. S.S. Mookerjea for the opportunity to do this research. His continued support and advice have been educational and inspiring. I am especially indebted to Dr. A. Nagpurkar for his valuable suggestions throughout. I am grateful to Dr. K.M.W. Keough for helpful discussions and critically reading the thesis and to Dr. V.S. Ananthanarayanan for helpful discussions.

I partly completed the work reported in Chapter 5 in the laboratory of Dr. P.J. Dolphin, Department of Biochemistry, Dalhousie University, Halifax, and I thank him for many stimulating discussions while there. My sincere thanks to Dr. Y. Legal, Dr. J.A. Barrowman and Ms. Jennifer Hall, Health Sciences Centre, Memorial University, for cooperation and assistance with the plasmapheresis experiments. Dr. J.E. Coe, Rocky Mountain Laboratories, N.I.H., Hamilton, generously supplied the Syrian hamster sera.

I would like to express my sincere appreciation to all the members of the Biochemistry Department for creating a stimulating atmosphere and especially to Dr. G. Herzberg for the use of his computer to perform statistical analysis. Thanks are also due to Mrs. Shobhitha Ratnam, Mrs. Joan Collins and Mr. Mohan Sarkar for creating a pleasant working atmosphere in the lab, with special thanks to Mrs. Joan Collins for her assistance with the plasmapheresis experiments.

I wish to acknowledge the assistance of Mr. Douglas Hall, Department of Biochemistry, in performing the amino acid analysis. I appreciate the very efficient assistance of Miss Carol Murphy in the preparation of the thesis.

I wish to thank Dr. F.A. Aldrich, Dean, School of Graduate Studies for his encouragement and financial assistance in the form of a Memorial University Graduate Fellowship. I am grateful to the Canadian Heart Foundation for the award of a Graduate Research Traineeship from 1983 to 1986.

I thank many friends for their interest in my progress. Finally I thank my parents for their love and support and I dedicate this thesis to them.

PUBLICATIONS

Much of the work presented in this thesis has been published or submitted for publication as full papers. These papers are:

- Nagpurkar, A., Saxena, U. and Mookerjea, S. (1983) Interaction of rat serum phosphorylcholine binding protein phospholipid-containing liposomes. J. Biol. Chem. 258, 10518-10523.
- Saxena, U., Nagpurkar, A. and Mookerjea, S. (1985) Contrasting effect of phosphorylcholine binding protein from rat and rabbit on heparin-lipoprotein interaction: a role of sialic acid. Can. J. Biochem. Cell. Biol. 63, 1014-1021.
- Saxena, U., Nagpurkar, A., Coe, J.E. and Mookerjea, S. (1986) The role of female hamster protein (FP) on the interaction between serum lipoproteins and heparin. Biochem. Cell. Biol. (Submitted).
- Saxena, U., Nagpurkar, A., Dolphin, P.J. and Mookerjea, S.S. (1986) A study on the selective binding of apo B and apo E containing human plasma lipoproteins to immobilised rat serum phosphorylcholine binding protein. J. Biol. Chem. (Revised and submitted).
- Saxena, U., Nagpurkar, A. and Mookerjea, S. (1986) Inhibition of the binding of low density lipoproteins to liver membrane receptors by rat serum phosphorylcholine binding protein. Biochem. Biophys. Res. Commun. (Submitted).

- Mookerjea, S., Saxena, U. and Nagpurkar, A. (1986) Interaction between phosphorylcholine binding proteins and serum lipoproteins. Prot. Biol. Fluids 34 (In press).
- Mookerjea, S., Saxena, U., Coe, J.E. and Nagpurkar, A. (1986) Role of sialic acid present on phosphorylcholine binding proteins on heparin-lipoprotein precipitation reaction. J. Biosciences (submitted by invitation).

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

Apo	Apoprotein or apolipoprotein
BSA	Bovine serum albumin
CD	Circular dichroism
CPS	C-polysaccharide
CRP	C-reactive protein
d	density
DES	Diethylstilbesterol
EDTA	Ethylenediamine tetraacetic acid
FP	Syrian hamster female protein
HDL	High density lipoproteins
IDL	Intermediate density lipoproteins
LCAT	Lecithin cholesterol acyl transferase
LDL	Low density lipoproteins
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
MW	Molecular weight
PAGE	Polyacrylamide gel electrophoresis
PCBP	Rat serum phosphorylcholine binding protein
PC	Phosphatidylcholine
P-choline	Phosphorylcholine/Ca^{2+} salt
SAP	Serum amyloid P-component
SDS	Sodium dodecyl sulfate

CHAPTER I

INTRODUCTION - A REVIEW

Lipids when transported in plasma exist as soluble complexes with specific proteins termed as apolipoproteins or apoproteins (apo). The lipid-protein complexes in plasma are termed as lipoproteins. The function of lipoproteins is to transport hydrophobic lipids in the aqueous environment of plasma from the tissues that synthesize lipids to the tissues that utilize them. The transport function and metabolic fate of lipoproteins depend primarily on the structure of lipoproteins and the interactions they undergo in the physiological milieu with other cellular and extracellular macromolecules. In this thesis, studies are presented on the interaction of lipoproteins with a phosphorylcholine binding protein (PCBP) from rat serum. Therefore, the aim of this review is to briefly introduce the reader to the field of plasma lipoproteins. In addition, studies on the interaction of lipoproteins with glycosaminoglycans have been included which are relevant to the work reported in this thesis. Finally, major points of information on the structure and properties of C-reactive proteins - a family of proteins with ligand specificity towards phosphorylcholine, have been presented.

1.1. Overview on Plasma Lipoproteins

A. General physicochemical properties

Lipoproteins represent the third most abundant group of extracellular macromolecules in the circulation of humans, only albumin and gamma globulins being present in greater amounts. Amongst the plasma proteins, lipoproteins have a uniquely low density. Depending on relative proportions of lipid and protein in the lipoprotein, the size and hydrated density of lipoproteins vary considerably. Plasma lipoproteins were first separated and classified according to their differential electrophoretic migration on solid support media into those remaining at the origin; beta- , pre beta- and alpha migrating species (Table 1) (1).

Lipoproteins are now most commonly isolated on a large scale by sequential ultracentrifugal floatation at increasing density solutions of sodium chloride or sodium bromide (2). There are four major classes of lipoproteins, isolated at different densities viz., the chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and the high density lipoproteins (HDL). These are considered to represent the major lipoprotein species in humans (3). Within each class, subpopulations of particles have been recognized when the more commonly used floatation techniques were complemented with density gradient fractionation, affinity and column chromatography, and electrophoretic methods. In normolipidemic plasma, at least two subclasses

Table I

Classification of plasma lipoproteins

Lipoprotein class	Density g/ml	Diameter (A°)	Molecular weight x 10^6	Electrophoretic mobility
Chylomicrons	<0.94	750-10000	10^3-10^4	origin
VLDL	0.94-1.006	550	5.27	pre-beta
IDL	1.006-1.019	250	4.75	beta
LDL	1.019-1.063	200	3.95	beta
HDL_2	1.063-1.125	110	0.22	alpha
HDL_3	1.125-1.21	88	0.16	alpha

Adapted from Dolphin (4).

of VLDL have been identified. The LDL class has been divided into two main subclasses, intermediate density lipoproteins (IDL) of d 1.006-1.019 g/ml and LDL proper of d 1.019-1.063 g/ml. Of the HDL's, the best defined subclasses by flotational criteria are HDL_2 (d 1.063-1.120) and HDL_3 (d 1.120-1.210 g/ml). The average chemical composition of the various lipoprotein species is shown in Table 2. Moving across the Table 2 from chylomicrons to HDL_3, the contents of protein, cholesteryl ester and phospholipids increase, whereas the triglyceride content decreases. Thus the chylomicrons and the VLDL are relatively triglyceride rich whereas approximately half of the lipid in HDL particles is phospholipid. The major constituent of the phospholipids in the lipoproteins is phosphatidylcholine (PC). In addition to the above mentioned major lipoprotein species in humans, Berg (5) has described another minor lipoprotein, Lp(a) lipoprotein, which is present in varying amounts in most normal individuals. Lp(a) lipoprotein is cholesterol-rich and contains the sialic acid rich Lp(a) antigen.

B. Apolipoproteins

Studies conducted during the last several years have shown that in addition to the solubilization of hydrophobic lipids in the aqueous environment of the plasma, the apoproteins have important metabolic functions. The major biosynthetic sites, the molecular weights and the various ascribed functions

Table 2

Chemical composition of plasma lipoproteins

(percent by weight)

	Lipoprotein					
	Chylomicron	VLDL	IDL	LDL	HDL_2	HDL_3
Protein	2	8	17	20.5	46	61
Triacylglycerol	90	55	29.5	5.1	2.5	1.9
Cholesterol	1.0	7.0	5.8	8.5	3.6	1.9
Cholesteryl ester	3.0	12.0	22.7	42.3	18.8	14.2
Phospholipids	4.0	18.0	24.2	23.6	28.1	20.0
Carbohydrate	< 1	< 1	< 1	< 1	1.0	1.0

Adapted from Dolphin (4).

of the major apoproteins are presented in Table 3. The nomenclature of apoproteins introduced by Alaupovic (6) is most widely used and has been followed in this thesis. At least five major apoproteins namely A, B, C, D and E have been characterized. The A apoproteins, apo A-I, A-II and A-IV are three distinct proteins predominantly found in the HDL density range in plasma and are also associated with chylomicrons (Table 3). Apoprotein B (apo B) is the major apoprotein of LDL and is also present in VLDL and chylomicrons. Apo B is extremely insoluble in aqueous buffers after the lipids have been removed from the lipoprotein. It is sensitive to oxidation and proteases. Handicapped by these difficulties, even the estimated molecular weight of this apoprotein is uncertain. Recently, Kane et al. (7) have shown that apo B is heterogeneous and exists primarily as two forms: apo B-100 a higher molecular weight form and apo B-48 the lower molecular weight form. Apo B-100 is synthesized exclusively by the liver whereas apo B-48 is synthesized by intestine alone. The C apoproteins (apo C) are represented by three low molecular weight proteins designated as apo C-I, C-II and C-III. These apoproteins are components of chylomicrons, VLDL and HDL. Apo D, also termed as apo A-III by Kostner (8), is associated with HDL. Apo E is a constituent of chylomicrons, VLDL and HDL. The amino acid sequences of apo A-I, A-II, C-I, C-II, C-III and E are also known (9). Apo A-II, B, C-III, D and E are glycoproteins (4).

Table 3

Biosynthetic Site, Distribution and Function of Apoproteins

Apoprotein	Molecular Weight (daltons)	Major biosynthetic site	Distribution	Function
AI	28,331	Intestine, liver	HDL_2, HDL_3	Activation of LCAT
A-II	8,707(x2)	Intestine, liver	HDL_2, HDL_3, lymph chylomicron	Activation of hepatic lipase
A-IV	46,000	Intestine	HDL, lymph, chylomicrons, d > 1.21 gm/ml	Activation of LCAT
B-100	549,000	Liver	VLDL, LDL	Secretion of VLDL, receptor binding
B-48	264,000	Intestine	Chylomicrons	Chylomicron secretion
C-I	6,500	liver	Chylomicrons, VLDL, HDL	Activation of LCAT, LPL activator?
C-II	8,837	Liver	Chylomicrons, VLDL, HDL	Activation of LPL
C-III	8,240	Liver	Chylomicrons, VLDL, HDL	Inhibition of LPL
D (A-III)	32,500	?	HDL_2, HDL_3, d>1.21	?
E	34,145	Liver	Chylomicron, VLDL, HDL_2	Receptor binding, activation of LCAT

Taken from Dolphin (4).

C. Structure of plasma lipoproteins

Based on experimental and theoretical considerations several models of lipoprotein structure have been proposed. A commonly held concept, supported by physical and chemical data, is that plasma lipoproteins are spherical or quasispherical particles having a core of triglycerides and/or cholesteryl esters stabilised by a monolayer composed of phospholipids, cholesterol and specific apoproteins (3). A large body of experimental evidence favours the concept that apoproteins play an essential role as determinants of lipoprotein structure. An important step in the definition of specific mechanisms by which apoproteins perform their structural function has been the establishment of the primary structure of most of the apoproteins.

Segerest et al (10) and Jackson et al. (11) have proposed a model for HDL based on a lipid-protein interaction hypothesis, known as the amphipathic-helix theory. This model is consistent with X-ray scattering data. An increase in the α-helical content of the apoprotein occurs, when apoproteins bind with phospholipids (3). In an attempt to explain this association of apoproteins with phospholipids, Segerest et al (10) proposed that the apoproteins form a unique structure which allows their interaction with phospholipids. The outstanding feature of the α-helical segments of the apoproteins is that they are amphipathic. One face of the helix is extremely hydrophobic and interacts with the carbons C_2-C_4 of the

phospholipid fatty acyl chains. The opposite face of the helix is hydrophilic and contains acidic amino acids near the centre which are frequently paired with basic amino acid residues at the edge of the helix. The authors postulate that there may be ionic interactions between the pairings of acidic and basic residues and the zwitter-ionic polar head groups of phospholipids. With this unique arrangement, the apoproteins can present a polar surface to the aqueous environment of the polar head groups of the phospholipids and the plasma, and a non-polar surface to the hydrophobic fatty acyl chains of phospholipids.

Assman and Brewer (12) have proposed a model for HDL which places particular emphasis on the hydrophobic interactions between polypeptides and the fatty acyl chains of phospholipids and views the apoproteins as icebergs immersed in a sea of lipids. This model attributes only a minor role to the ionic interactions between apoprotein and phospholipids.

The importance of hydrophobic interactions between phospholipids and apoprotein in HDL is also emphasized in the model proposed by Stoffel et al. (13) which rules out the occurrence of strong electrostatic interactions involving the polar head groups of phospholipids and the polar surface of the apoproteins.

The first model that attempts to consider all the lipoproteins with a common structure has been proposed by Shen et al. (14). They inferred the structure from compositional

analysis of the lipoproteins and found good correlation between size and chemical composition of the lipoproteins. According to this 'hydrophobic lipid core hypothesis', both the apoproteins and the hydrophilic head groups of phospholipids are closely packed at the outer surface of the lipoprotein. Cholesterol interacts directly with the protein but is not exposed to the aqueous environment. The hydrophobic core consists of triglycerides and cholesteryl esters which is surrounded by the monolayer of protein, phospholipid and cholesterol. Phospholipids and protein compete for space on the surface of the lipoproteins. A model for the general structure of lipoproteins proposed by Shen et al. (14) is shown in Fig 1.

D. Mechanisms of formation, secretion and degradation of lipoproteins

Two organs have been shown to synthesize plasma lipoproteins, the liver and the intestine. Chylomicrons originate from the intestine as a consequence of the dietary intake of fat and enter the circulation through the lymphatic system. In the circulation, there is a rapid transfer of apo C-II, C-III and E from HDL to chylomicrons. These apoprotein additions are accompanied by a loss of apo A-I, A-IV and phospholipids which appear to be transferred to plasma HDL fraction (15,16). The acquisition of apo C-II, a specific activator of the enzyme lipoprotein lipase (LPL),

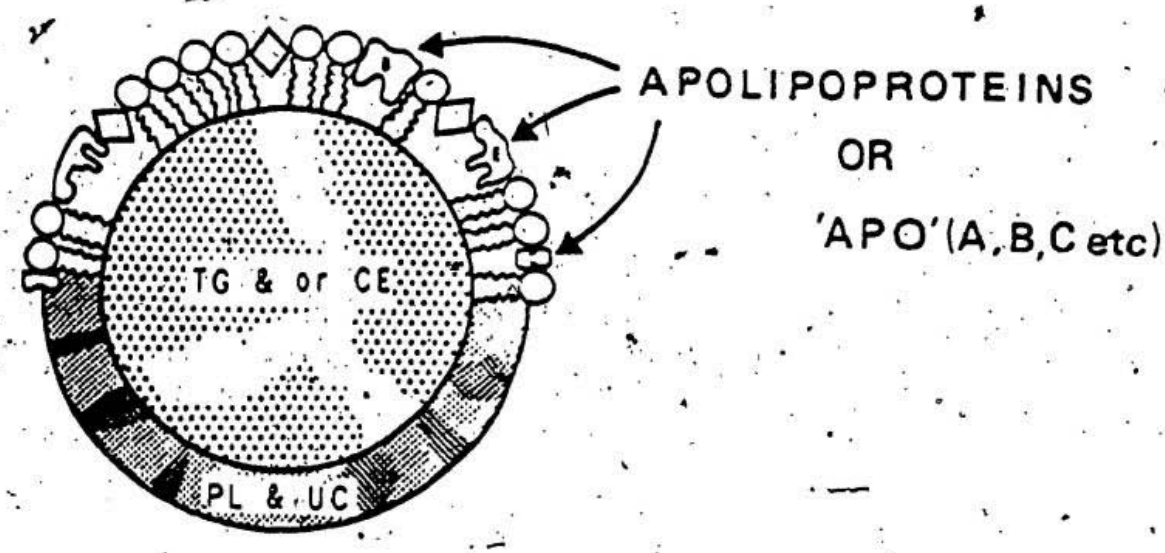


Fig. 1. The general structure of plasma lipoprotein particle. The structure is based on the hydrophobic lipid core hypothesis (14). This hypothesis predicts that most of the hydrophobic lipids (TG and CE) occupy the central core of the particle with the relatively less hydrophobic lipids (UC and PL) forming the surface monolayer. TG, triglycerides; CE, cholesteryl esters; PL, phospholipids, UC, cholesterol. (Adapted from Dolphin (4)).

permits LPL to hydrolyse 20-30% of the core triglycerides. During the course of chylomicron triglyceride hydrolysis, apo C-II and C-III are lost from the chylomicron surface and transferred back to the HDL fraction (15). The fatty acids released on triglyceride hydrolysis are taken up by tissues (muscle and adipose) for oxidative metabolism or storage. The chylomicrons, with their core triglycerides depleted, form smaller particles which have been termed as chylomicron 'remnants' (16). The presence of apo E on these remnant particles, mediates their clearance by liver cells via a receptor termed as apo E or chylomicron remnant receptor (17,18).

VLDL is formed in the liver and the metabolic transformations of VLDL upon entry into the plasma compartment are similar to those of chylomicrons. Apo C-II and C-III are acquired from plasma HDL. In addition, apo E content of VLDL is supplemented by transfer from HDL (15). The core triglycerides of VLDL are hydrolysed by LPL resulting in the formation of a smaller IDL particle (19). During this process apo C-II and C-III are lost from VLDL to HDL fraction. Excess surface phospholipid and cholesterol are transferred mainly to HDL fraction and small amounts of phospholipid and cholesterol may form bilaminate vesicular structures (19,20). The IDL particles thus generated are converted to LDL, a process mediated by LPL as well as another enzyme called hepatic lipase (15,20). During this process the residual

apo C and majority of apo E from IDL are lost to HDL fraction, resulting in formation of LDL, whose major apoprotein is apo B-100. Some of the IDL is removed from the plasma by the apo E receptor and the LDL receptor. The LDL receptor is discussed below.

A major discovery has been the recognition of the so-called LDL-receptor pathway, which accounts for the uptake and degradation of LDL by the peripheral cells and liver. According to the studies pioneered by Goldstein and Brown (21), the critical component of this mechanism is a high affinity receptor, i.e. the LDL receptor or apo B, E receptor, localized on the plasma membranes of cells. Apo B-100 and apo E have been shown to mediate the interaction of lipoproteins with this LDL receptor. The binding of LDL sets in motion a series of events by which LDL is taken up by the cells through absorptive endocytosis, transferred to the lysosomes, and hydrolyzed by acid hydrolysis. This process of LDL uptake and lysosomal degradation leads to the suppression of cholesterol synthesis through modulation of the activity of the enzyme 3-hydroxy-3-methylglutaryl Co-A reductase, an increase in the esterification of cholesterol by the enzyme acyl Co-A cholesterol acyl transferase and a decrease in the number of LDL receptors expressed on the cell surface. Thus uptake of LDL by the receptor-mediated process permits cells to acquire cholesterol from the lipoprotein and this in turn not only provides sterol for membrane synthesis but also for

cellular regulatory actions that prevent over-accumulation of cholesterol in the cells.

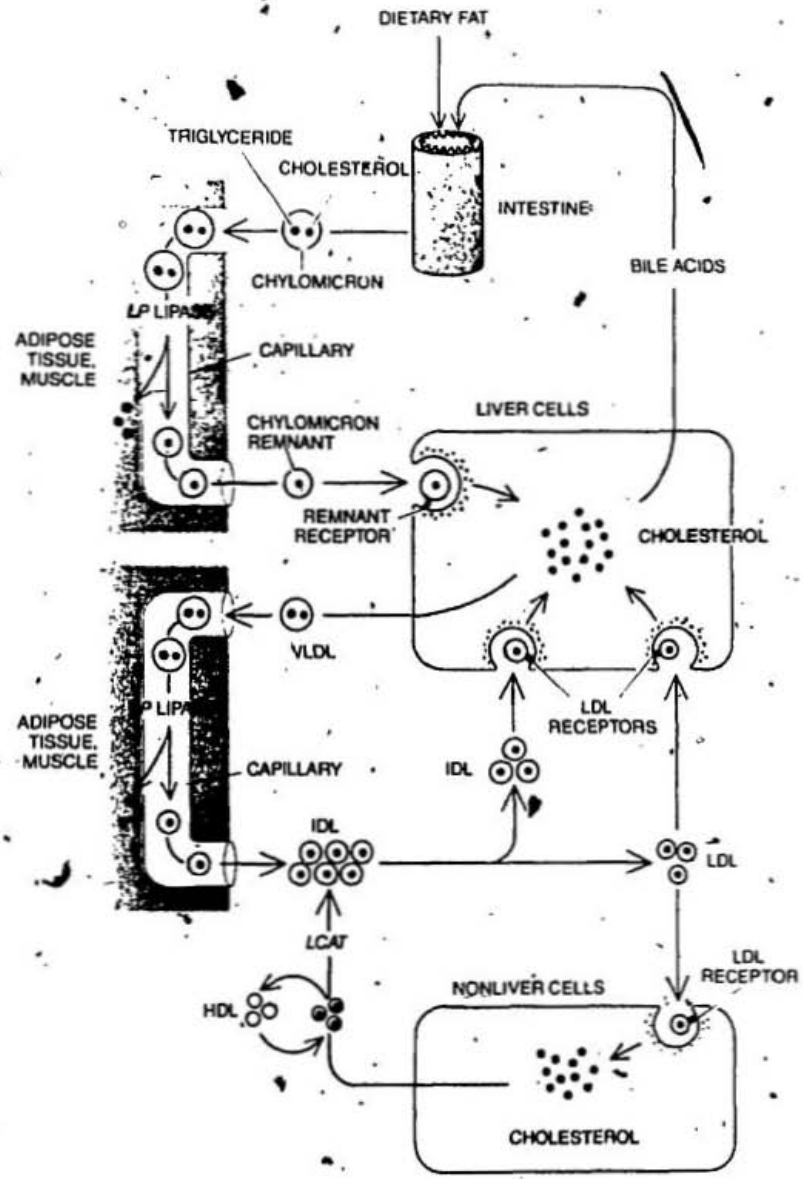
LDL carry the bulk of plasma cholesterol in man and elevated levels of plasma LDL are correlated with increased risk of atherosclerosis (21,22). The importance of LDL receptors in regulating plasma LDL levels has been confirmed by the observation that genetic absence (or defective functioning) of LDL receptor results in premature onset of atherosclerosis (23,24).

As mentioned above, apo E-containing lipoproteins are also capable of interacting with the LDL receptor and regulating cholesterol metabolism (25). This has been shown with several subclasses of HDL that do not contain apo B but do contain apo E. These subclasses are HDL_1 and HDL_C. Selective chemical modification of arginine or lysine residues abolish the ability of LDL and HDL_C to interact with LDL receptors on cultured fibroblasts (26,27). These studies demonstrated the importance of a limited number of arginine and lysine residues of apo B and apo E in mediating the receptor-lipoprotein interactions.

The biosynthesis of HDL also occurs in both the intestine and the liver (28). There is strong evidence indicating that HDL is secreted as a 'nascent' precursor from these organs which is discoidal in structure and consists of phospholipids, unesterified cholesterol and apoproteins (Apo A-I or apo E) but is deficient in cholesteryl esters. The

Fig. 2. General scheme of plasma lipoprotein metabolism.

Dietary triglyceride and cholesterol enter the circulation in the form of chylomicrons formed in the intestine. In the capillaries of adipose and muscle tissue, the triglyceride core is markedly reduced through the action of enzyme LPL and the fatty acids are removed. The cholesterol-enriched remnants are removed by liver through the hepatic remanant receptors (apo E receptors). Their cholesterol is either secreted as bile acids into intestine or packaged with triglyceride to form VLDL. VLDL is secreted into the circulation and the triglyceride is hydrolysed by the enzyme LPC in adipose or muscle tissue leading to the formation of IDL. Some IDL is cleared by the liver through the LDL receptors (apo B, E receptors) and the remainder is transformed into LDL. Most of the LDL is taken up by liver or other tissues through the LDL receptors and thus cleared from the circulation. HDL acquires cellular cholesterol from peripheral cells which is rapidly esterified by the enzyme LCAT. A portion of the peripheral cell derived cholesterol esters may be transferred to VLDL or IDL and ultimately taken up by various cells (adapted from Brown and Goldstein (22).


DIETARY FAT
TRIGLYCERIDE
CHOLESTEROL
INTESTINE
CHYLOMICRON
BILE ACIDS
LP LIPASE
ADIPOSE TISSUE, MUSCLE
CAPILLARY
LIVER CELLS
CHYLOMICRON REMNANT
REMNANT RECEPTOR
CHOLESTEROL
VLDL
LDL RECEPTORS
ADIPOSE TISSUE, MUSCLE
CAPILLARY
IDL
IDL
LDL
LCAT
LDL RECEPTOR
NONLIVER CELLS
HDL
CHOLESTEROL

maturation of discoidal HDL requires cholesterol esterification which is mediated by the lecithin-cholesterol acyl transferase (LCAT) enzyme reaction (28). LCAT acts specifically on plasma HDL by converting the lecithin and unesterified cholesterol of HDL to cholesteryl esters and lysolecithin (29,30). Once esterified, the free cholesterol leaves the surface coat and moves into the non-polar lipid core in the center of the particle, leading to the transformation of disc shaped 'nascent' HDL into spherical 'mature' HDL. Apo A-I, the principal apoprotein of HDL, is an activator of the reaction (31).

In addition to their direct biosynthesis in the liver and intestine, the discoidal structures have been postulated to form as products pinched off from chylomicron and VLDL surface as the hydrolysis of core triglyceride takes place (19,20). In addition to the function of HDL to serve as a reservoir for apoproteins, it is now believed that the HDL-LCAT system is involved in the reverse transport of cholesterol as cholesteryl ester from peripheral tissues to the liver for utilization and ultimate excretion of cholesterol in the bile (32). According to this concept, HDL and in particular the nascent phospholipid-apoprotein discoidal structure can acquire unesterified cholesterol from peripheral tissues and esterification of this cholesterol by LCAT leads to the formation of mature HDL. The newly formed cholesterol esters now occupy the particle core of HDL and from there are

transferred to LDL and VLDL and finally cleared by the liver (4). HDL that contain apo E may be directly removed by the hepatic apo E receptor and HDL that contain predominantly apo A-I can interact with cells via the hepatic apo A-I receptor (4,32). In keeping with the role of HDL in cellular cholesterol egress, an extensive literature has accumulated supporting a negative correlation between plasma HDL levels and incidence of cardiovascular disease (33,34).

In addition to the enzyme-mediated processes mentioned above, specific protein-mediated transfer of lipids between lipoprotein species has also been shown. In the lipoprotein-free fraction ($d > 1.21$ g/ml) of human plasma, a protein termed as cholesteryl ester transfer protein has been demonstrated that mediates the exchange of core cholesteryl esters of each lipoprotein, especially from HDL to VLDL. It has also been postulated that such a process is accompanied by back transfer of triglycerides from VLDL to HDL (35,36). In addition a protein that may facilitate the transfer of phospholipids between lipoproteins has been reported (37). This phospholipid transfer protein from human plasma has been shown to mediate the transfer of phospholipids from vesicles of egg lecithin to HDL. The purified protein has a molecular weight of 41,000 (37).

From the above discussion, it is apparent that a set of mechanisms is operative in controlling the synthesis of lipoproteins, their secretion into blood stream, and their

subsequent clearance. The net consequence of these processes is the delivery of lipids to tissues while the lipoproteins acquire the appropriate surface structure to interact with cells and mediate control of their cholesterol production and secretion. It therefore becomes relevant to study the interactions of lipoproteins with various plasma constituents and cells in order to gain insight into the hoemostatic controls of circulating lipoproteins.

During the past few years considerable advances have been made in the study of plasma lipoprotein in various animal species. Selective aspects of lipoprotein structure and metabolism in rats will be discussed here, since it has relevance to the work described in this thesis.

In most studies, rat plasma lipoproteins have been isolated by ultracentrifugation and designated using the density ranges established for human lipoproteins. The rat has lipoproteins equivalent to all the major lipoprotein species in human plasma. The major plasma lipoprotein species of the rat are HDL which carry approximately 75% of the plasma cholesterol (38). In addition to the HDL_2, the rat has another HDL-like lipoprotein called HDL_1 (39). Both HDL_2 and HDL_1 in rats contain apo E in substantial amounts and it is the major apoprotein constituent of HDL_1.

In describing the rat plasma apoproteins, investigators have applied the nomenclature developed for human apoproteins. Rat apo E has (70%) homology of amino acid sequence with

human apo E (40). In addition rat apo C-I and C-II also appear to be homologous to their human counterparts based on similarities in amino acid composition (41). The primary structure of rat apo A-I is very similar to that of human apo A-I (42). Rat apo A-IV has also been described (43). Unlike the human organs, both the rat intestine and liver synthesize apo B-48 (44).

There are two major differences in the metabolism of lipoproteins between rat and human. The rat possesses an efficient mechanism for clearance of VLDL from the circulation due to interaction with the hepatic apo E receptor. This rapid clearance is attributed the presence of both apo B-100 and B-48 in rat VLDL. The apo B-48 component, which is the major proportion of the VLDL apo B is rapidly cleared from the plasma and only a portion of B-100 forms LDL (45,46). This causes low levels of LDL in rat plasma, which may account for the resistance of the rat to the development of atherosclerosis compared to humans.

The second major difference is the absence of cholesteryl ester exchange protein in rat plasma as a result of which there is accumulation of cholesteryl esters in HDL and a relative paucity of these molecules in other lipoproteins (47).

1.2. Lipoprotein-glycosaminoglycan interactions

A. Introduction

The glycosaminoglycans are characteristic components of

vertebrate connective tissues (48). They are long, unbranched carbohydrates which contain many acidic (sulfate or carboxyl) groups. They do not normally occur as free polysaccharide chains *in vivo*, but as proteoglycans in which many chains are linked at the terminal reducing sugar residue to a protein molecule. Glycosaminoglycans are primarily molecules of extracellular space and they thus occur in greatest abundance in those tissues in which the extracellular space is large. These are often tissues with a structural function such as cartilage, cornea, skin and blood vessel walls. In other 'soft' tissues such as liver, kidney and brain glycosaminoglycans are also present, but in smaller amounts.

There are seven different types of glycosaminoglycans (namely, hyaluronic acid, chondroitin 4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparin sulfate and heparin) that are commonly found in vertebrate tissue (48). They are all long unbranched polysaccharides in which the chain is made up of disaccharide repeating units consisting of a hexosamine and a hexuronic acid. The amino groups of the hexosamine residues are generally substituted with N-acetyl groups, but can also contain N-sulfate groups in heparin and heparin sulfate. The presence of sulfate and/or carboxyl groups on each disaccharide unit makes the chain strong polyanions and this dominates to a large extent their interaction with other molecules. In this section information on the interaction of glycosaminoglycans with plasma lipoproteins

will be reviewed.

In 1955, Burstein and Samaille (49) showed that heparin forms insoluble complexes with serum lipoproteins in the presence of divalent cations. Even in the absence of divalent cations, heparin increased the electrophoretic mobility of β-lipoprotein (LDL), thus showing the formation of a soluble complex with an increased negative charge (50). LDL and other serum proteins did not form any complex in the presence of 0.01% heparin. Subsequently Burstein and Scholnick (51) showed that by a judicious use of different divalent cations and by suitable adjustment of the concentration of heparin, chylomicrons, VLDL and LDL can be sequentially precipitated. The differential precipitation of lipoproteins with divalent cations has been used for quantitative estimation of particular lipoprotein classes in plasma (51). The interactions between various glycosaminoglycans and lipoproteins in vitro have been studied by a number of techniques. The early studies by Bernfeld utilised nephlometery and free boundary electrophoresis (50,52). Iverius (53) has studied the binding of lipoproteins to glycosaminoglycan immobilized on agarose. In addition these interactions have also been studied by gel filtration chromatography (54) and by precipitation techniques (55). Calcium induced precipitation has been widely used in measuring the interaction of lipoproteins with heparin by turbidimetric assays (55). These assays are based on the formation of insoluble lipoprotein-heparin-

calcium complexes.

B. Nature of lipoprotein-glycosaminoglycan interactions

The nature of interactions between lipoproteins and glycosaminoglycans has been investigated in various studies. The following discussion has been limited to selected studies that outline the current understanding of glycosaminoglycan-lipoprotein interactions with particular emphasis on studies that utilised heparin.

Iverius (53) investigated the interaction of lipoproteins with heparin immobilised on Sepharose. The apo B containing lipoproteins VLDL and LDL bound to heparin-Sepharose in the absence of divalent cations, whereas HDL did not bind. The binding of VLDL or LDL to heparin-Sepharose could be abolished by N-acetylation of the lysine residues of the apoproteins, suggesting an important role for the lysine residues. Since both VLDL and LDL but not HDL were bound to heparin-Sepharose, it was proposed that apo B in part was responsible for binding of VLDL and LDL to heparin.

However, HDL subfractions that contain apo E as the exclusive or predominant apoprotein, namely canine HDL_C (56), a fraction of rat HDL (57) and a fraction of human HDL (58) have been shown to bind to heparin-Sepharose. In their studies with canine HDL_C, Mahely et al. (56) reported that acetylation and carbamylation of apo E prevented the binding of HDL_C to heparin-Sepharose thus demonstrating an important

role for apo E in the binding.

The studies of Shelbourne and Quartfort (59) have also shown the importance of apo E in the binding of VLDL to heparin. VLDL subfractions containing no apo E were shown not to bind to heparin-Sepharose at physiological ionic strength and in the absence of divalent cations. Apo E isolated from whole lipoprotein was treated with phenylglyoxal to modify the arginine residues and this modified apo E was not able to bind to heparin-Sepharose.

Pan et al. (54) have studied the formation of a soluble complex in the absence of divalent cations between heparin and LDL by gel filteration chromatography. No binding of HDL or VLDL to heparin could be demonstrated. It was further demonstrated that modification of histidine, lysine or arginine residues on the apoprotein resulted in a loss in heparin binding.

On the basis of the studies mentioned above, it would appear likely that the interaction of lipoproteins with heparin involves the basic amino acid groups in the apoproteins (apo B and E). Iverius (53) postulated that the lipoprotein-glycosaminoglycan complex formation is primarily caused through ionic interactions between basic amino groups of the apoprotein and the anionic sulfate groups of glycosaminoglycans (53).

Several studies (60,61,62) have also examined the involvement of the phospholipid moiety of lipoproteins in the interaction with heparin and other sulfated polysaccharides.

Treatment of LDL with phospholipase C was shown to interfere with its precipitation with heparin in the presence of Ca^{2+}. The formation of insoluble complexes decreased as the degree of hydrolysis of phospholipids increased (60). Girard and Canal (61) extracted phospholipids from human serum and showed interaction of the phospholipids with heparin in the presence and absence of Ca^{2+}. In the presence of Ca^{2+}, the soluble phospholipid-heparin complexes were converted into insoluble precipitate.

Kim and Nishida (62) have shown that in the presence of Ca^{2+}, high molecular weight dextran sulfate (molecular weight, 150,000) precipitates lecithin dispersions and lysolecithin micelles prepared from egg yolk. The precipitation was proposed to arise from two mutually enhancing interactions involving both positive and negative charges of the zwitterionic phospholipids, with one resulting from the interaction between the positive charge of the nitrogen base in lecithin and the sulfate negative charge of dextran sulfate, and the other from Ca^{2+} cross linking of phosphate groups of neighbouring phospholipids. The above mentioned studies suggest that phospholipids are involved in the interaction of heparin with lipoproteins in the presence of Ca^{2+}.

On the basis of the observations discussed in preceeding paragraphs and other evidence, it is generally agreed that the interactions between lipoproteins and glycosaminoglycans have following features: a) In the absence of divalent cations,

the sulfated groups of glycosaminoglycans interact primarily with the basic amino groups of apoproteins (apo B and E) to form soluble complexes; b) Independent of this, the addition of divalent cations can bridge the sulfated groups of glycosaminoglycans and lipoprotein phospholipid, thereby promoting the aggregation and insolubility of the complexes (Fig. 3).

C. Functional significance of lipoprotein-glycosaminoglycans interaction.

The functional possibilities of the interactions between lipoproteins and glycosaminoglycans include a role in the development of atherosclerosis (63,64). The most likely mechanism by which plasma lipoproteins can be selectively retained in arteries is by ionic binding. The fact that glycosaminoglycans can form complexes *in vitro* with the atherogenic LDL and VLDL but not HDL (except apo E containing HDL) makes such putative interactions very important for the sequestration of plasma lipoproteins within the artery.

Recently, attention has been focussed on the surface charge of LDL in terms of atherogenesis (65,66). In the arterial wall, an electrostatic interaction of LDL with glycosaminoglycans may make the lipoprotein particles more electronegative. Unlike arterial smooth muscle cells, macrophages bind and internalize only electronegative LDL by a high affinity process lacking in feedback control (65). Such a

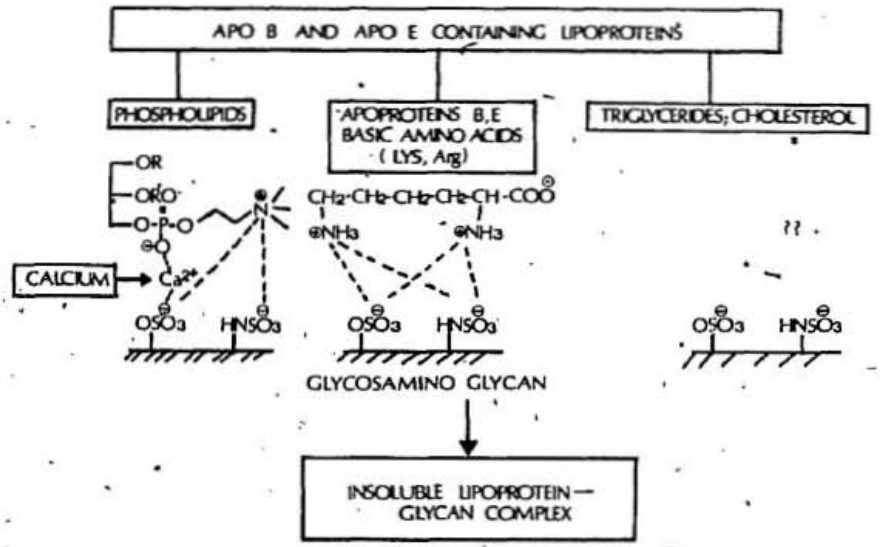


Fig. 3. Schematic illustration of the mechanism of interaction between glycosaminoglycans and plasma lipoproteins in the presence of Ca^{2+}.

As proposed by various authors (53,63,64), electrostatic interactions govern the formation of insoluble lipoprotein-glycosaminoglycan complexes. The sulfated groups of glycosaminoglycans interact with the basic amino groups of lysine and arginine residues present in apo B and E. The positive charge of the phospholipid choline nitrogen interacts with the sulfate negative charge of the glycosaminoglycan. The formation of insoluble lipoprotein-glycosaminoglycan complex is enhanced by Ca^{2+} cross-linking of the phospholipid phosphate groups to the sulfate groups of glycosaminoglycans.

process has been proposed to result in the formation of fat-filled foam cells present in atherosclerotic lesions. It is of particular interest that exogenous sulphated glycosaminoglycans administered to animals have alleged anti-atherosclerotic effects (63,67). This behaviour has been attributed to their controlling the interaction of atherogenic lipoproteins with arterial glycosaminoglycans.

More recently, interest has been focussed on the interaction of apo B and apo E containing lipoproteins with heparin because of parallels between heparin binding and the interaction with cell surface LDL receptors (56,68). Heparin has been shown to compete with LDL receptors for binding with LDL and has been used to displace LDL bound to LDL receptors (69). Furthermore, there appears to be a correlation between precipitability of LDL and HDL_c with heparin and the binding of LDL and HDL_c to receptors (70). The interaction of lipoproteins with both the LDL receptors and heparin appears to involve similar basic amino acids present on apo B and apo E (56).

1.3. C-reactive protein and its homologs

A. Introduction

The acute phase response consists of the increased production of a number of plasma proteins which occur following most forms of tissue injury, inflammation, infection and malignant neoplasia. The C-reactive protein (CRP) was one

of the first acute phase proteins to be identified. During the acute phase response, the serum concentration of CRP may increase 1000 fold or more (71). CRP was discovered in humans by Tillet and Francis (72) in 1930 as a material that precipitated with pneumococcal C-polysaccharide (CPS). Abernethy and Avery (73) then characterized human CRP as a protein and identified the requirement for calcium in its interaction with CPS. CPS is a heteropolymer consisting of N-acetyl-galactosamine, D-glucose, N-acetyl-diaminotri-deoxyhexose, ribitol and phosphorylcholine (74) (Fig. 4). The binding of human CRP with CPS has been shown to be mediated by the phosphorylcholine moiety of CPS (76). Since its discovery in the sera of patients with various infections and inflammatory diseases, CRP and its homologs have been identified in various mammalian species, in certain fish and even invertebrates (Table 4). The known specificity of human CRP for phosphorylcholine in the presence of Ca^{2+} has been utilized to purify the various proteins homologous to human CRP using either an immobilized CPS, phosphorylcholine or phosphorylcholine derivative affinity adsorbents. Another protein in man, called serum amyloid P component (SAP) has been shown to be related to CRP (77). It is now believed that CRP and SAP belong to a unique family of proteins know as "pentraxins" (77). Human CRP is composed of five subunits and SAP contains ten subunits. The latter differs from CRP in that it is not an acute phase protein and it is glycosylated.

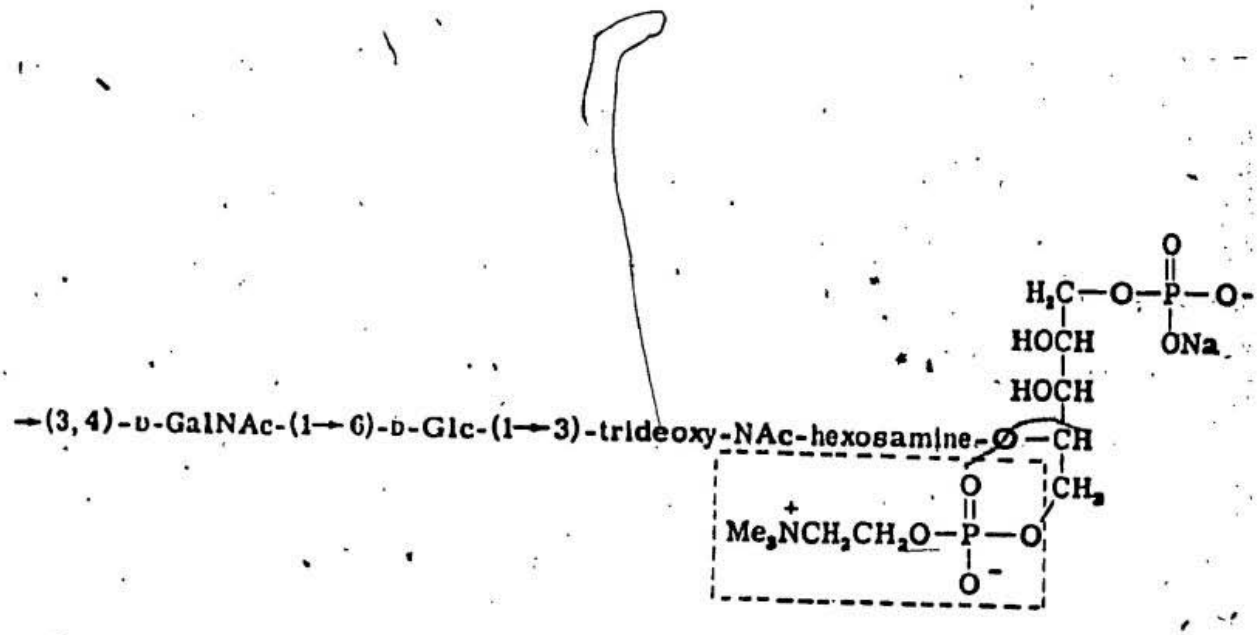


Fig. 4. Structure of Pneumococcus C polysaccharide.

Brundish and Baddiley (74) reported that Pneumococcal C polysaccharide is composed of N-acetyl-D-galactosamine, D-glucose, N-acetyl-diaminotrideoxyhexose, ribitol and phosphorylcholine. The dashed lines indicate the phosphorylcholine unit which is the primary binding site for human CRP (76). (Adapted from Glaudemans (75).

Table 4

Molecular Properties of C-reactive proteins

	Human CRP (71,77)*	Rabbit CRP (82,78)	Plaice CRP (85)	Dogfish CRP (86)	Limulus CRP (87)
Molecular weight (daltons)	110,000	115,000	300,000	250,000	500,000
Subunits	5	5	10	10	12
Association of subunits	non-covalent	non-covalent	non-covalent	covalent (disulphide)	non-covalent
Glycosylation	-	-	+	-	+
Level in normal serum (mg/ml)	Trace	Trace	0.05-0.06	0.4	5
Induction due to acute phase, (fold)	1000	1000	small	?	?

*The numbers within the parentheses indicate the source of information.

The definition of a protein as a member of the pentraxin family depends on the structure of subunits by electron microscopy. The name pentraxin is derived from the observation that the subunits of both CRP and SAP are arranged in annular discs with cyclic pentameric symmetry (77). The determination of a CRP or SAP homology is currently determined by the ligand binding capacity of the protein. A pentraxin is termed as 'CRP' if it possesses Ca^{2+} dependent phosphorylcholine binding specificity and is termed as 'SAP' if it binds to agarose in a calcium-dependent manner (78).

Besides the pentameric symmetry of its subunits, human SAP also shares substantial (51%) amino-acid sequence homology with human CRP (79). CRP and SAP are believed to be products of gene duplication (78). The structural genes for both have been mapped to chromosome 1 (80). SAP is a precursor of amyloid P component which is found in basement membranes and associated with amyloid deposit (81). In this section some pertinent literature on the structure, ligand binding and biological properties of human CRP and its homologs will be reviewed.

B. Structure and ligand binding

Table 4 shows some of the properties of human CRP and homologous proteins described in various vertebrates and invertebrates. Human and Rabbit CRP consist of five non-glycosylated identical subunits which are non-covalently

associated, with evidence for the existence of an intrachain disulphide bridge within each subunit (82,83,84). Plaice (Pleuronectes platessa L.) CRP is exceptional in that it contains non-covalently associated subunits of two different molecular weights of which only one type is glycosylated (85). Dogfish (Mustelus canis) CRP has been isolated and purified (86) and its molecular properties are presented in Table 4. The invertebrate homolog of CRP ie. Limulus CRP has been isolated from the haemolymph of horseshoe crab (Limulus polyphemus) using phosphorylcholine affinity adsorbent (87). It consists of two types of non-identical subunits with molecular weights in the range of 18,000 and 24,000. Both types of subunits are glycosylated. Unlike the human and rabbit CRP, Limulus CRP is a major normal haemolymph component at a concentration of approximately 5 mg/ml (87).

The Ca^{2+} dependent binding specificity of human CRP for phosphorylcholine was first demonstrated by Volanakis and Kaplan (76) from their studies on the inhibition of CRP-CPS precipitation reaction. Gotschlich and Edelman (88) showed that each of the five subunits of human CRP molecule probably binds two Ca^{2+} ions after which CRP binds one mole of phosphorylcholine per subunit. The ligand best bound by human CRP is phosphorylcholine (76) but, other phosphate monoesters are also bound with a stoichiometry of 1 mole per CRP subunit although with much lower affinity (89). Young and Williams (90) studied the ability of a number of small

molecular weight compounds to inhibit the human CRP-CPS interaction and emphasized the specificity for phosphate monoester group. For instance choline base-sulfonate or sulfate were not inhibitory. It was also found that increasing the space between the phosphate and trimethylammonium group by one carbon decreased the inhibitory power by two fold. Oliveira et al. (91) based on their studies of human CRP with BSA conjugated with phosphorylcholine or phosphorylethanolamine proposed the idea that two loci on each subunit of CRP are involved in the binding of phosphorylcholine. Their results showed that the binding site of CRP consists of two loci: a primary locus responsible for the calcium dependent binding of phosphorylester moiety of phosphorylcholine and a secondary locus for binding the cationic group of phosphorylcholine. In another study, the binding of phosphorylcholine by human CRP was shown to be inhibited by certain di and tripeptides provided they contained both a free carboxyl and a free α-amino group thus confirming the zwitterionic nature of the binding site (92).

The studies mentioned above suggest the specificity of the binding site on human CRP for phosphorylcholine. However, studies comparing the precipitation of human and rabbit CRP with BSA-phosphorylcholine and BSA-phosphorylethanolamine suggest a major difference in the binding properties of human and rabbit CRP (89). For human CRP, precipitation required both a phosphate group and either a secondary or tertiary

amino group at appropriate spacing, while with rabbit CRP only Ca^{2+} mediated binding of phosphate group was obligatory for precipitation. The interactions of human and Limulus CRP with spin labelled derivatives of phosphorylcholine have been studied using electron spin resonance spectroscopy (93). It was suggested that the phosphorylcholine binding sites on both human and Limulus CRP are quite shallow, not exceeding 5A° in depth.

The role of Ca^{2+} in the binding of phosphorylcholine has been explored in a number of studies (88,90,94,95). Results indicate that Ca^{2+} induces a conformational change in human CRP which is necessary for it to express its characteristic phosphorylcholine binding property (88,90). The existence of Ca^{2}-induced conformational changes in human CRP have been confirmed using monoclonal mouse anti-human CRP antibodies (94). Potempa et al. (95) have reported that, in the presence of Ca^{2+} human CRP resists denaturation by heating or by high concentrations of urea. All these results indicate that Ca^{2+} induces conformational changes in human CRP which may be required for it to bind phosphorylcholine.

The other major binding property of human CRP appears to be its ability to bind polycations such as poly-L-lysine and poly-L-arginine polymers, lysine and arginine rich histones, myelin basic protein and leukocyte cationic protein (77). It has been shown that in the absence of Ca^{2+}, CRP and polycation form complexes at appropriate concentrations

leading to aggregation and precipitation (96). These interactions were inhibited by Ca^{2+} but addition of phosphorylcholine in the presence of Ca^{2+} promoted the interactions (97). The interactions between CRP and polycations were also modulated by heparin (98). It has been suggested that Ca^{2+} may be responsible for modulation between the phosphorylcholine and polycation binding reactions. The polycation binding site on CRP is proposed to be within or close to the phosphorylcholine binding site (77,96).

C. Amino acid sequence and assignment of phosphorylcholine binding region.

Important advances have been made in determining the amino acid sequence of CRP's from some species. The complete amino acid sequence of human CRP has been determined by sequencing the protein and also as derived from complementary DNA and genomic DNA nucleotide analysis (79,83,99).

The complete amino acid sequence of rabbit CRP has been elucidated (84) and partial amino acid sequence of Limulus (87) and plaice CRP is also available (85).

Based on the sequence data, extensive homologies are noted between human and rabbit CRP. Rabbit CRP has 70% identity to human CRP residue for residue (84). Comparison of the sequences derived for human, rabbit and Limulus CRP with SAP and the myeloma proteins (a group of phosphorylcholine binding immunoglobulins) has permitted the assignment of amino

acid residues that may be involved in the binding of phosphorylcholine. Since the sequence Phe 33 - Tyr 34 - Met 35 - Gly 36 present in the first hypervariable region of the heavy chain is common to phosphorylcholine binding myeloma proteins, Young and Williams (90) have suggested that in CRP, a similar sequence, Phe 39 - Tyr 40 - Thr 41 - Glu 42 may be involved in binding phosphorylcholine. They postulated that the phosphorylcholine binding site in CRP may consist of Glu 42 and Glu 66 for the positively charged quarternary ammonium ion and Lys 57 and Arg 58 for the negatively charged phosphoryl group respectively.

The sequence in the proposed binding site for phosphorylcholine also revealed important variation between human CRP and rabbit CRP. Glu 42 in human CRP has been replaced by Asp in rabbit CRP, Lys 57 in human CRP has been replaced by Phe in rabbit and Limulus CRP. Therefore, Wang et al (84) suggest that since Glu 66 in human CRP is not conserved in other CRP, it is not likely to be involved in the binding of positively charged group of the ligand.

It is of interest to note that Woo et al. (79) in their search for homologies of CRP with proteins other than SAP in the amino acid and nucleotide databases have suggested 28% homology between the first 25 amino acids of human CRP and snake phospholipase A_2 and human apolipoprotein CII.

Glu 61, which is conserved in all CRP species and also in human SAP together with Glu/Asp at position 42, was

proposed to be involved in binding calcium ion (84). On the other hand by analogy with other calcium binding proteins it has been suggested that tyr residues may be involved in the binding of Ca^{2+} by CRP (90).

D. Biological properties of CRP

Numerous properties of human CRP have been described which may bear relationship with the biological function of this protein. The elevation of CRP levels in serum in response to infection/inflammation suggests that its function may be related to host defense and repair and this suggestion has provided the rationale of much of the work.

i. CRP and the complement system

Following tissue damage in rabbits, CRP has been shown to be deposited on necrotic cells but not normal cells (100,101). Similarly, CRP deposition in experimental allergic encephalomylitis in rabbits has been investigated and CRP was detected in some of the spinal cord lesions, the amount of CRP correlating with the proportion of polymorphonuclear leukocytes (77).

There have been reports of CRP deposition in inflammatory conditions in man (77). The deposits were found in the cutaneous lesions containing mainly neutrophils of certain patients with vasculitis and also in association with synovial cell nuclei in synovial biopsies of patients with rheumatoid

arthritis.

Kaplan and Volanakis (102) were the first to report the activation of human complement by CRP-CPS complexes. It was shown that addition of CPS to CRP containing acute phase sera resulted in consumption of complement. The activation of complement pathway was completely inhibited by phosphorylcholine. Volanakis (103) has shown that human CRP does not bind to the intact membranes of living cells but binds to damaged or altered cells. In addition it has been shown that CRP can bind to liposomes and this binding then activates the complement pathway. It was demonstrated that the binding of CRP to lipid emulsions consisting of either phosphatidylcholine or sphingomyelin with cholesterol led to complement fixation. Further evidence for the involvement of human CRP in complement activation comes from the work of Gewurz and coworkers as discussed below, who not only showed the CRP initiated complement activation but also demonstrated that ligands that were able to induce complement activation included a wide variety of polycations and polycation-polyanion complexes mentioned below.

Complement activation by the classical pathway occurred when protamine was added to acute phase serum or when CRP and protamine were added to normal serum (104). Other polycations shown to react with CRP and consume complement included homopolymers of L-lysine and arginine, histones, myelin basic protein and cationic proteins from leukocyte

granules (105). CRP was shown to enhance complement activation by polyanions like DNA, hyaluronic acid and chondriotin sulfate (106).

Richards et al. (107,108) demonstrated that human CRP could activate human complement upon binding to liposomes consisting of dimyristoyl phosphatidylcholine, cholesterol and galactose ceramide when appreciable amounts of strong positive charge (stearylamine) was also incorporated. Increased cholesterol in the liposome and the presence of ceramide lipids enhanced complement activation (109). The ability of CRP to initiate complement dependent haemolysis was shown using sheep erythrocytes coated with CPS (110).

ii. Interaction of CRP with phagocytic cells and lymphocytes

Human CRP has been shown to bind to human monocytes and mediate phagocytosis (111). Using erythrocytes as particles for uptake by human monocytes, it has been shown that CRP mediates phagocytosis of CPS-coated erythrocytes sensitized with CRP in the presence of complement. Studies with mouse macrophages indicated that CRP may interact with a membrane receptor, FcR, or a closely related receptor on monocytes (112). This conclusion was reached on the basis of observations that CRP-CPS complexes inhibited the uptake of IgG coated erythrocytes by monocytes and that a selective inhibitor of FcR activity, 2-deoxyglucose, blocked the effects of CRP-CPS complexes on macrophages. These in vitro effects of CRP

were demonstrable in vivo in a mouse model in which CRP promoted the sequestration of CPS-coated erythrocytes by spleen. This effect required both the complement and the presence of CRP (113).

Since CRP binds to CPS of Streptococus pneumoniae, the ability of CRP to bind to bacteria and activate complement system has also been tested (113). It was shown that CRP binds to Streptococus pneumoniae Type 27 which contains phosphorylcholine in its capsular polysaccharide and enhances complement consumption. Using a mouse model, it has been shown that administration of human CRP provides protection against pneumococcal infection (113). This effect was seen both in mice that lacked circulating antibodies to phosphoryl-choline and in normal mice.

Studies on the interaction between human CRP and peripheral blood lympocytes show that CRP by itself does not bind to the lymphocytes but requires the addition of a ligand such as CPS whereupon CRP binds to a subset of lymphocytes bearing the FcR receptors (114). Prefetential binding to FcR bearing lymphocytes was also demonstrable when lymphocytes were reacted with heat-modified CRP. The effects of binding of CRP complexes on lymphocyte functions such as natural killer activity were investigated and CRP complexes or CRP has been shown to have no effect on this activity (114). However, treatment of human lymphocytes with anti-CRP antibody and complement has been shown to abolish some aspects of natural

killer function (114).

iii. Interaction of CRP with platelets

Fiedel and Gewurz (115) initially reported the ability of CRP to inhibit platelet responsiveness to a wide variety of platelet activators. Subsequently a low molecular weight factor, co-eluting with human CRP isolated from pleural or ascites fluids using CPS or phosphorylcholine affinity adsorbents was shown to be responsible for the inhibition of platelet aggregation and release reactions (116). In the absence of low molecular weight factor, CRP did not inhibit platelet activation. The inhibitory capacity of CRP-low molecular factor was substantially reversed in the presence of CPS. It was further shown that heat-modified CRP-activated platelets to elicit reactions of shape change, aggregation and release of dense granule constituents. In addition, heat-modified CRP also activated platelets to generate thromboxane A_2. Interestingly, complexes of CRP and polycations caused similar activation of platelets (117). More recently (118) it has been reported that peptide fragments derived from the degradation of CRP are associated with the platelet inhibitory activity.

There are two reports which suggest that human and rabbit CRP can inhibit platelet-activating factor (PAF) induced aggregation of platelets (119,120). PAF, a potent platelet aggregating agent, is a phosphorylcholine containing lipid

(121).

iv. Interaction of rabbit CRP with chromatin

In a recent study, rabbit CRP was shown to bind to isolated chromatin in the presence of calcium (122). Furthermore, CRP precipitated nucleosome core particles from chicken erythrocytes. In addition, it has been shown that human CRP mediates the solubilisation of chromatin by complement in vitro (123). Based on these results it has been postulated that CRP may mediate the removal of chromatin fragments from the body after cell death by complement mediated phagocytosis.

V. Summary.

It appears from a variety of studies that human CRP can mediate complement activation, enhance phagocytosis, modulate platelet activity and scavenge chromatin particles after cell death. All these properties are mediated through the ligand binding specificity for phosphorylcholine and polycations. These studies point to a role of CRP in host defense during injury and inflammation, a role in keeping with its effects upon complement, platelet and phagocytic systems.

E. Syrian hamster female protein

A novel protein termed as female protein (FP) was isolated by Coe (124) from the serum of Syrian hamsters. FP was originally noted because it was a prominent protein only

in female sera, but not in male sera. Subsequently FP was shown to be a member of the pentraxin family of proteins (125). However this protein has certain unique characteristics in comparison to other members of the pentraxin family and hence deserves separate discussion.

i. Structure and ligand binding specificities

FP has been shown to have similarities with both human CRP and SAP. It is composed of five subunits, (molecular weight 30,000 of each subunit) held together by non-covalent bonds and exhibits the pentagonal structure typical of the pentraxins (126). Like CRP, FP binds to phosphorylcholine affinity adsorbent and requires Ca^{2+} for this binding. Not only does FP possess phosphorylcholine binding specificity but it also binds to Sepharose in a Ca^{2+} dependent manner like SAP. However, the affinity of FP for phosphorylcholine is greater than Sepharose. The phosphorylcholine binding site on FP is believed to be involved in the interaction with Sepharose (126).

The amino acid composition of FP was similar to that of human CRP and sequence analysis of 23 of the first 26 residues from the amino terminus showed 83% homology with SAP. The protein was shown to be glycosylated (127). Recently a complementary DNA clone corresponding to FP was isolated and used to determine the nucleotide sequence and derive the amino acid sequence of FP (128). The amino acid sequence of FP was

shown to be 69 percent identical to human SAP and 50 percent identical to human CRP.

Although the amino acid sequence of FP is more similar to human SAP than to human CRP, FP has been shown to bind to phosphorylcholine (126). On the other hand FP has been shown to be constituent of hamster amyloid deposits, a property usually associated with SAP (126).

From the discussion presented above FP appears to be a homolog of both human CRP and SAP. However based on the current criteria adopted for nomenclature i.e. a pentraxin that shows Ca^{2+}-dependent binding to phosphorylcholine being classified as homologous with CRP, FP would be defined as a CRP homolog. Its amino acid sequence however suggests closer relationship with SAP. Therefore it appears that determination of CRP or SAP homology cannot be based on binding criteria alone.

iii. Hormonal regulation of FP

FP is the only pentraxin which has been shown to be under stringent sex hormonal control (126). The serum level of FP in adult female syrian hamsters is about 1-2 mg/ml whereas the serum of adult male syrian hamsters contains about 100 fold less. The low level in male was suggested to be due to supression of FP by testosterone, since it was inducible after treatment with diethylstilbesterol (DES) or by castration.

F. Rat serum phosphorylcholine binding protein

In 1973, Mookerjea, (129) while seeking the function of choline and phosphorylcholine in lipoprotein metabolism investigated the possible role of phosphorylcholine in the interaction between heparin and lipoprotein in the presence of Ca^{2+}. These efforts led to a discovery that phosphorylcholine in minute amounts plays a role to accelerate the precipitation of rat serum lipoproteins by heparin and Ca^{2+} (129). Studies conducted to test the specificity of the effect of phosphorylcholine on heparin-lipoprotein precipitation reaction showed that choline and a number of choline derivatives, inorganic and organic phosphates, amino and neutral sugars had no effect on the precipitation reaction. These results suggested the importance of phosphorylated quaternary nitrogen group in promoting the heparin-lipoprotein precipitation reaction in rat serum.

Further investigations were carried out to gain insight into the specific enhancing effect of phosphorylcholine on heparin - rat serum lipoprotein precipitation (130). Attempts to show the effect of phosphorylcholine on heparin-lipoprotein precipitation using purified rat chylomicrons and VLDL proved unsuccessful, which suggested that the phosphorylcholine effect was related to the use of total serum. The effect of phosphorylcholine in promoting the interaction between heparin and pure chylomicrons and VLDL was regained when a crude serum protein factor of unwashed chylomicrons was

added to the assays, suggesting that rat serum contains a protein factor(s) which normally inhibits the heparin-lipoprotein precipitation reaction. A number of further trials showed that a protein fraction of rat serum prepared by precipitation with 30-50% ammonium sulphate inhibited heparin-VLDL and heparin-chylomicron precipitation reactions in a concentration dependent manner. The inhibitory effect of the protein fraction was relieved when phosphorylcholine was added to the assays. From these results it became apparent that rat serum contains a protein-factor which can inhibit heparin-lipoprotein precipitation reaction and addition of phosphorylcholine counteracts the inhibitory effect of this protein factor.

On the basis of the specific effect of phosphorylcholine on this protein factor, a ligand binding specificity of the factor towards phosphorylcholine was anticipated. Subsequently, Nagpurkar and Mookerjea (131) purified the protein from rat serum using a phenyl phosphorylcholine-Sepharose affinity adsorbent. The protein bound to the affinity adsorbent was eluted by phosphorylcholine, but could not be eluted using acetylcholine or choline chloride. On the basis of its ligand binding property for phosphorylcholine, the protein was termed as phosphorylcholine binding protein (PCBP). The molecular weight of purified PCBP was found to be 125,000 by gel-chromatography on Sephacryl S-300. The molecular weight of the protein was also determined by the sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and it separated into two bands of molecular weight 47,000 and 24,000, attributable to a monomer and dimer of the same unit. Reduction with 2-mercaptoethanol resulted in dissociation of the dimer, giving rise to a single band of molecular weight 24,000. From these observations it was concluded that PCBP consists of five subunits, three of which are held together noncovalently while the other two are covalently bonded through a disulfide bridge. Amino acid analysis showed that each subunit consists of 145 amino acids and the protein was found to contain 18% carbohydrate. The carbohydrate moiety consists of glucosamine, galactosamine, hexosamine, neutral hexoses and sialic acid. PCBP was found to be present in normal rat serum in substantial amounts (0.6-0.7 mg/ml).

Purified PCBP when added to heparin-lipoprotein precipitation assays using purified rat chylomicrons or VLDL inhibited the precipitation reaction and addition of phosphorylcholine reversed the inhibitory effect of PCBP. Antibody to PCBP raised in rabbits produced a single precipitin line against the pure antigen thus confirming the immunochemical identity of the isolated protein obtained from rat serum. However, the antiserum failed to produce any precipitin line against rat chylomicron, VLDL, LDL and HDL thereby suggesting that PCBP has no immunochemical identity with the apoproteins of the serum lipoproteins. The antiserum also failed to cross react against human CRP, nor did PCBP cross react with

the anti-human CRP.

Binding studies using the equilibrium dialysis technique showed that PCBP binds 3 mol of phosphorylcholine per mol of protein in the presence of Ca^{2+} (132). The synthesis and secretion of PCBP by the liver was shown using an isolated hepatocyte system (133). Tunicamycin, an antibiotic which inhibits the glycosylation of the core linkage region of N-glycoproteins inhibited its synthesis.

Subsequent to the isolation of PCBP by Nagpurkar and Mookerjea (131), Pontet et al. (134) and De Beer et al. (135) isolated an identical protein from rat serum and termed it as SAP and rat CRP respectively. The criteria utilized for naming the protein as rat CRP by De Beer et al (135) was its binding specificity towards CPS (78). Similarities with human CRP were noted, i.e., pentagonal structure when examined by electron microscopy and amino acid sequence homology with CRP, although only 30% of the amino acids from the carboxyl terminal were sequenced and showed 71 percent homology with human CRP and 54 percent homology with SAP (136).

While the amino acid sequence analysis, which by itself is incomplete, supports the relationship of this protein to human CRP, however, certain abberant features have also been noted. Unlike human or rabbit CRP, PCBP is glycosylated and is present as a prominent constituent of normal rat serum (131). In addition, rat serum PCBP/CRP, unlike human CRP, fails to activate the complement pathway and does not agglutinate

or precipitate soluble CPS (78). Complete amino acid sequence analysis together with the use of molecular biological techniques may establish a more meaningful homology with human CRP at the protein and genetic level. Based on its pentagonal structure, PCBP has been classified as a member of the pentraxin family (126).

1.4. Purpose of present research.

On the basis of the observation that PCBP has an inhibitory effect on heparin-lipoprotein precipitation reaction and has a specificity for phosphorylcholine ligand, it seemed reasonable that understanding the molecular basis of binding properties of PCBP with phospholipids and plasma lipoproteins would shed light on the function(s) of this protein. Elucidation of its function in lipoprotein metabolism may also advance the knowledge of other members of the pentraxin family.

In this thesis previous studies on the role of PCBP in heparin-lipoprotein interactions have been extended. A comparison of PCBP with effects of other pentraxins, human and rabbit CRP, hamster FP on heparin-lipoprotein precipitation reaction has been made. The role of sialic acid residues on PCBP and FP in heparin-lipoprotein precipitation reactions has been investigated. These studies may throw light on the nature and mechanism of the effect of PCBP and other pentraxins on heparin-lipoprotein interactions.

A systematic study on the binding of PCBP to phospholipid

containing liposomes has been undertaken to gain insight into the nature and specificity of PCBP-phospholipid interactions.

The binding of human plasma lipoproteins to immobilized PCBP has been characterized in order to identify the basic features involved in the interaction of PCBP with plasma lipoproteins. The characterization of the lipoprotein bound and unbound to immobilized PCBP with respect to apoprotein and lipid composition may help in understanding the nature and specificity of PCBP-lipoprotein interactions.

The effect of PCBP on interactions between membrane LDL receptors and LDL has been investigated to explore the possible functional effect(s) of PCBP-lipoprotein interaction. Finally the ability of Sepharose-PCBP columns to bind lipoproteins when used in an in vivo experimental plasmapheretic system using rabbit model has been tested to evaluate the extent to which plasma VLDL and LDL can be removed by Sepharose-PCBP columns.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

A. Animals

i) Rats

Male Sprague-Dawley rats (body weight 250-300g) were obtained from Charles River Canada Inc., La Praire, PQ and were fed purina rat chow ad libitum (Ralston Purina of Canada Ltd., Don Mills, Ontario).

ii) Rabbits

Male white New Zealand rabbits (body weight 1.5-2.5 kg) were purchased from Charles River Canada Inc., La Praire, PQ and were fed rabbit chow ad libitum (Robinhood Multifoods Inc. St. John's).

B. Reagents and Chemicals

Unless otherwise specified, reagents and chemicals were of commercial origin and best reagent grade available.

C. Serum and Plasma

i) Rat serum

Rats were anesthesized lightly with ether, the abdomen opened and blood obtained from the abdominal aorta. The blood was allowed to clot for 1 hr at room temperature followed by another hour at 4°C, then centrifuged for 15 minutes at 1000xg on a bench top centrifuge to obtain the serum. Serum was used immediately or stored at -20°C until use.

ii) Rabbit serum

Blood was obtained by cardiac puncture from rabbits under anesthesia induced by intraperitoneal injection of sodium phenobarbital, 65 mg/kg body weight, from M.T.C. Pharmaceuticals, Hamilton, Ontario. Serum was prepared as described above for rats.

iii) Human Plasma

Fresh, rejected plasma from normal donors prepared with citrate phosphate dextrose as an anti-coagulant, was obtained from Canadian Red Cross Society, St. John's and used within 24 hrs for isolation of lipoproteins. In some cases human plasma was prepared from freshly drawn venous blood of healthy human subjects. Blood was drawn into heparinized tubes and plasma prepared by low speed centrifugation.

iv) Hamster sera

Sera from male, female, FP-depleted female, diethylstilbestrol (DES) treated male, and testosterone-treated female syrian hamsters were supplied by Dr. J.E. Coe, NIH, Hamilton, Montana. The sera which had been sent by air, packed in dry ice were used immediately on arrival or stored at $-20^{o}C$.

D. Antiserum

Antiserum to PCBP raised in rabbits was available from a previous study (131). Antiserum to rabbit CRP raised in goats was a gift from Dr. V. Cabana, Department of Immunology, Rush Medical College, Chicago. Antiserum to Lp(a) and apo C

II were made available by Dr. W.C. Breckenridge, Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia.

E. Human CRP and SAP

Purified human CRP and SAP were purchased from Difco Laboratories and Calbiochem respectively.

2.2. Treatment of animals

A. Induction of inflammation

Inflammation was induced in both rats and rabbits by the subcutaneous injection of commercial grade turpentine (rats: 0.5 ml/100g body weight; rabbits: 0.5 ml/kg body weight) in the dorsolumbar region, equal volumes being injected either side of the midline. The control group was injected in an identical fashion with 0.9% saline. Both groups were bled, 48 hours after injection, and serum prepared. The serum from inflamed rabbits was checked for the presence of CRP by immunodiffusion analysis using antiserum to rabbit CRP.

B. Administration of 17α-ethinylestradiol to rats

17α-ethinylestradiol (Sigma) was dissolved in propylene glycol at a concentration of 10 mg/ml and administered subcutaneously to rats at a dose of 3 mg/kg body weight every 24 h for seven days (137).

C. Induction of hypercholesterolemia in rabbits by cholesterol diet

A 2% cholesterol (w/w)–5% coconut oil (w/w) diet was prepared by dissolving cholesterol (Sigma) in hot coconut

oil and mixing it with rabbit-chow. Rabbits were maintained on this diet for periods ranging from 1 to 4 weeks before use in plasmapheresis experiments. Control diet consisted of ordinary rabbit chow.

2.3. Preparative Procedures

A. Preparation of Sepharose-phenylphosphorylcholine affinity adsorbent.

The affinity adsorbent for chromotography of phosphorylcholine binding proteins was prepared as described by Nagpurkar and Mookerjea (131). Typically, 4-nitrophenylphosphorylcholine (0.6g, 1.19 nmol from Sigma) in methanol was reduced with H_2 at 1 atm using 0.2g of 5% Palladium on charcoal as catalyst for 2 hours at room temperature. The reaction mixture was filtered and the filtrate evaporated under reduced pressure. The resulting 4-aminophenylphosphorylcholine was immediately dissolved in 0.1M $NaHCO_3$ buffer (pH 8.3) containing 0.5M NaCl and added to 15g of cyanogen bromide (CNBr) activated Sepharose 4B (Pharmacia) which had been previously washed with 1 mM HCl. The mixture was gently mixed for 4 hours at room temperature, filtered and then washed alternately with buffers 0.1M $NaHCO_3$ (pH 8.3) containing 0.5M NaCl and 0.1M CH_3COONa (pH 4.2) containing 0.5M NaCl. The Sepharose-phenylphosphorylcholine was treated overnight at 4°C with 1M ethanolamine (pH 9.0) and finally resuspended in 5 mM Tris-HCl (pH 7.8). Typically, the concentration of phosphorylcholine incorporated was 26 µmol/ml of Sepharose.

B. Purification of pentraxins

i) PCBP

PCBP was purified from normal serum and serum from inflamed rats according to the procedure of Nagpurkar and Mookerjea (131). Sepharose-phenylphosphorylcholine affinity adsorbent was packed in a column (12x1.6 cm) and equilibrated with 200 ml of 5 mM Tris-HCl buffer (pH 7.8). Normal serum or serum from inflamed rats (15 ml) was brought to a concentration of 5 mM with respect to Tris-HCl and applied on the affinity adsorbent. The column was washed with equilibration buffers until absorbance at 280 nm of the eluant was less than 0.02. The serum eluted by this washing represents the PCBP-depleted serum. Thereafter, after exhaustive washing the bound PCBP was eluted with 50 ml of 4 mM P-choline in the same buffer. The eluted protein was exhaustively dialyzed against the equilibration buffer (4 changes, 50 volumes) and stored at -20°C. The quantitative estimation of PCBP in serum was performed as described (131). A known aliquot of the serum was chromatographed on the affinity adsorbent. The bound PCBP was eluted by applying a P-choline gradient (0-0.1M) and estimated by the method of Lowry et al. (138). Quantitative depletion of PCBP from the serum was established by immunodiffusion analysis using the antiserum. The purity of PCBP preparations for each batch was established by polyacrylamide gel electrophoresis (PAGE) according to the method of Davis (139).

ii) Rabbit CRP

Rabbit CRP was isolated from serum from inflamed animals following identical procedure used for rat PCBP using the affinity adsorbent (I). Rabbit CRP-depleted serum was also obtained in a similar fashion. The complete depletion of CRP from rabbit serum was established by immunodiffusion analysis using antiserum to rabbit CRP. The CRP isolated, was determined to be free of other proteins by analysis on PAGE (139). Purified CRP was stored at -20°C in 5 mM Tris-HCl (pH 7.5) at a concentration of less that 1 mg/ml.

iii) Hamster FP

FP was isolated from normal female hamster serum essentially as described by Coe et al. (125). Female hamster serum (2 ml) was applied to a column (0.7x50 cm) packed with 15 ml of Sepharose-phenylphosphorylcholine affinity adsorbent equilibrated with 0.1M Tris-HCl buffer (pH 7.0) containing 0.5 mM Ca^{2+}. The column was washed extensively with equilibration buffer and FP was eluted with a P-choline gradient (0 to 3.3 mM) in the equilibration buffer. FP eluted was estimated by the method of Lowry et al. (138), dialyzed extensively against 10 mM sodium phosphate (pH 7.0) containing 150 mM NaCl and stored at -20°C. The purity of FP was checked by PAGE (139).

C. Preparation of desialylated pentraxins

i) Desialylated PCBP

PCBP (10 mg; 80 nmol, based on MW 125,000) was incubated in 20 mL of 5 mM Tris-HCl buffer (pH 7.6) with 2.5U neuraminidase

(from Clostridium perfringens type X from Sigma; EC 3.2.1.18) at 37°C for 12h. The release of sialic acid was monitored using the method of Warren (140). After completion of the reaction, the incubation mixture was applied to the Sepharose-phenylphosphorylcholine affinity column (30 mL bed volume). The column was washed extensively with Tris-HCl buffer and the bound desialylated PCBP was eluted with a P-choline gradient (0-25 mM) in Tris-HCl buffer. The protein was dialysed against 5 mM Tris-HCl (pH 7.8) (4 changes, 50 volumes) and stored at -20°C.

ii) Desialylated FP

FP (3 mg; 20 nmol based on 150,000 MW) was desialylated by neuraminidase treatment by incubating in 10 ml of 5 mM Tris-HCl buffer (pH 7.6) with 1 unit of neuraminidase (E.C. 3.2.1.18) from Clostridium perfringens type=X at 37°C for 12h. Release of sialic acid was monitored using the method of Warren (140). At the end of the incubation, the reaction mixture was applied to Sephafose-phenylphosphorylcholine affinity adsorbent (15 ml bed volume, 0.7x50 cm) equilibrated with 0.1M Tris-HCl buffer (pH 7.0) containing 0.5 mM Ca^{2+}. The column was extensively washed with the equilibration buffer and the bound desialylated FP was eluted with a P-choline gradient (0-3.3 mM) in the same buffer. The protein was dialysed extensively against 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl and stored at -20°C.

D. Isolation of human plasma lipoproteins

Lipoproteins were isolated from 250 ml plasma units. Prior to isolation sodium azide was added to the plasma at a final concentration of 0.01% (w/v). Lipoproteins were isolated by sequential flotation in a Beckman L5-50B ultracentrifuge according to the method of Havel et al. (2), using a Beckman 60 Ti rotor. The plasma was adjusted to desired densities by the addition of solid NaBr.

Centrifugation was carried out in 36 ml pollyallomer quick seal tubes (Beckman) at 12°C. At the end of the centrifugation tubes were sliced with a tube slicer (Beckman), so as to obtain two fractions, the supernatant containing the isolated lipoprotein and the infarnate containing the remaining lipoproteins. The bottom fraction was then adjusted to desired density to obtain the next lipoprotein fraction. The top fraction containing the isolated lipoprotein was washed by recentrifugation at the appropriate density. The lipoproteins were isolated following the removal of chylomicrons (17,000 RPM, 30 min at 10°C). VLDL was isolated at d 1.006 g/ml (40,000 RPM, 18 h at 10°C). LDL was then isolated between densities 1.030 and 1.063 g/ml (45,000 RPM, 15 h at 10°C). HDL was isolated between densities 1.063 and 1.21 g/ml (50,000 RPM, 24 h at 10°C). Purified lipoprotein fractions were dialyzed for 24 h at 4°C against 3 changes of 50 volumes of 0.01M Tris-HCl buffer (pH 7.5) containing 0.15M NaCl and 0.01% (w/v) sodium azide. The protein content of the lipoproteins was measured by a modified method of Lowry using sodium

dodecyl sulfate (SDS) to clear turbidity (141). The lipoproteins were stored in sterile plastic tubes at 4°C and used within 3 weeks. The purity of the lipoproteins was ascertained by examining the apoprotein content on SDS-PAGE using 4-30% gradient gels (142).

E. Preparation of liver membranes from 17α-ethinyl estradiol treated rats

Liver membranes were prepared essentially as described by Kovanen et al. (137). Rats were killed by decapitation and the livers were immediately removed and placed in ice cold 0.15M NaCl. Equal aliquots from 5 livers were weighed and pooled for homogenization. All further steps were carried out at 4°C. One gram of pooled liver, was homogenized with two 10-sec pulses in a Polytron homogenizer (Brinkman Instruments, Setting No. 10) in 10 ml of homogenization buffer (150 mM NaCl, 1 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5). The whole homogenate was centrifuged at 500xg for 5 min. The resulting 500xg supernatant was centrifuged at 8000xg for 15 min, and the 8000xg supernatant was centrifuged at 100,000xg for 60 min. Each 100,000xg pellet was resuspended in 6 ml of homogenization buffer by flushing 10 times through a 22 gauge needle, and the suspension was again resedimented at 100,000xg for 60 min. The final pellet was assayed immediately or rapidly frozen in liquid nitrogen, stored at -80°C and used within 3 weeks.

F. Preparation of phospholipid containing liposomes.

Multilamellar liposomes were prepared to study the binding of PCBP to phospholipid containing liposomes. Egg PC, egg LPC, phosphatidylethanolamine, phosphatidylserine, synthetic dioleoyl- and dipalmitoylphosphatidylcholine, BSA, fatty acid-free BSA were purchased from Sigma and synthetic 1-oleoyl-2-palmitoyl phosphatidylcholine was a gift from Dr. K.M.W. Keough, Department of Biochemistry, Memorial University of Newfoundland.

Multilamellar liposomes were prepared by dissolving the phospholipids at the desired concentration in choloroform and evaporating the chloroform solution to dryness under nitrogen. The resulting lipid mixture was dispersed in 2 ml of 0.125M Tris-HCl buffer (pH 7.8), so as to give a final phospholipid concentration of 10 mM. The dispersion was vortexed for 3 min before using in the assay in order to produce an uniform suspension. Freshly prepared liposomes were always used in the assays.

G. Preparation of Sepharose-PCBP and Sepharose-desialylated-PCBP affinity adsorbents.

Purified PCBP was covalently linked to Sepharose in the following way: Five grams of CNBr activated Sepharose 4B (Pharmacia) were washed with 1000 ml of 1 mM HCl. Sepharose was then filtered almost to dryness and 18 mg PCBP in 25 ml of 0.1M sodium bicarbonate buffer (pH 8.3) containing 0.5M

NaCl were added. The mixture was gently mixed at 26°C and filtered after 2 hrs. The resulting Sepharose-PCBP was treated for 16 h at 4°C with 1M ethanolamine (pH 9.0). The protein content of the filtrate was measured (138) to determine the amount of PCBP not coupled to Sepharose. The amount of PCBP coupled to Sepharose was determined by subtracting the protein present in filtrate from total protein added for coupling. With this procedure, 1 mg of PCBP was coupled per ml of Sepharose. The coupling was tested by the ability of Sepharose-PCBP to bind antibodies to PCBP from the antiserum. Desialylated PCBP (prepared as described before) was coupled in an identical fashion to give a concentration of 1 mg protein per ml of Sepharose. Blank Sepharose was also prepared by treating CNBr activated Sepharose 4B as described above except that no protein was added for coupling.

2.4. Radioiodinitation procedures

A. PCBP

Radioiodinated PCBP was prepared enzymatically with lactoperoxidase and glucose oxidase using radioiodination system from New England Nuclear following the manufacturers instructions. A typical labelling reaction mixture included 0.168 mg PCBP in 100 µl of 5 mM Tris-HCl (pH 7.8) and 1 mCi of $Na^{125}I$. Unbound $Na^{125}I$ was removed by gel filtration chromatography on a sephadex G-50 column (20x1.2 cm). ^{125}I-PCBP was further purified by Sepharose-phenylphosphorylcholine

affinity chromotography. The ^{125}I-PCBP prepared by this method had a specific activity of 0.43 nmol of ^{125}I/ mol of PCBP.

B. Human plasma LDL

Human plasma LDL was radioiodinated by modification of the method of McFarlane (143) as described by Wong and Rubinstein (144). Na^{125}I (100μCi), Iodine monochloride (80 nmol), 1M glycine-NaOH buffer pH 10.00 (0.2 ml) and LDL (1 mg protein in 2 ml) were added sequentially. After 30-60 sec, the radioiodinated LDL was applied to a Sephadex-G25 column (1.5x26 cm) and eluted with 0.05M Tris-HCl buffer pH 7.4 to separate labelled LDL from free iodine. The ^{125}I-LDL protein concentration ranged from 0.25 mg to 0.32 mg/ml, the specific activity ranged from 121-249 dpm/ng protein. An average of 5% of the ^{125}I-radioactivity was extractable into ethanol-ether (3:1) and greater than 95% of the ^{125}I-radioactivity was precipitable by incubation with 10% (v/v) trichloroacetic acid. Labelled LDL was stored in sterile plastic tubes at 4°C and used within 2 weeks of preparation. Before each experiment ^{125}I-LDL was dialyzed overnight against 0.01M Tris-HCl buffer (pH 7.4) containing 0.15M NaCl to remove any residual free iodine.

2.5. Analytical Procedures

A. Circular Dichroism (CD)

CD spectra of PCBP and desialylated PCBP were

measured on a Jasco J-20 spectropolarimeter at 25°C. PCBP and desialylated PCBP samples were prepared in 5 mM Tris-HCl buffer (pH 7.6). The results are presented as mean residue ellipticities (θ) in degree.cm^2.$dmole^{-1}$, using the mean residue molecular weight of PCBP and desiaylated PCBP as 112.2, based on amino acids.

B. SDS-Polyacrylamide gel electrophoresis of apoproteins

Apoprotein content of lipoproteins was analyzed on SDS-PAGE using 4-30% gradient gels prepared according to the method of O'Farrel (142). Prior to electrophoresis, lipoprotein fractions were delipidated for 16h at -20°C with ethanol-ether (3:1 v/v). The delipidated apoproteins were dissolved in 0.2M Tris-HCl buffer (pH 6.8) containing 3.6% SDS and 10% 2-mercaptoethanol. The gradient gels were run at 30 mA per slab for 4 hours at constant amperage. Following electrophoresis, gels were fixed in ethanol-water-acetic acid and stained overnight in 0.025% Commassie brilliant-blue. Gels were destained by diffusion in 10% acetic acid. The apoproteins were identified by their designated molecular weights determined from calibration standards run together with the samples.

D. Electroimmunoassay of apoproteins

i) Apo B

Apoproteins were quantitated individually by an electroimmunoassay ('Rocket' electrophoresis) following the procedure of Laurell (145). The conditions and validation of

the assays for apo B, E and A-I have been described previously by Dolphin et al. (146).

The electrode buffer for the assay of apo B contained 0.06M sodium barbital buffer pH 8.6 with 1.5% polyethylene glycol 6000 (PEG-6000) (Sigma) and 0.0025M calcium lactate and was used also as the sample diluent. The electrophoresis was performed in 1% agarose (Seakem L.E., Marine Colloids, Rockland, ME) containing the electrode buffer and 50 g rabbit antihuman apo B gamma globulin per ml agarose. Electrophoresis was performed at 2.5 v/cm for 18 hours at 12°C using a Bio-Rad Model 1415 electrophoresis cell. After electrophoresis the plates were washed in distilled H_2O for 1 h, covered with Whatman No. 1 filter paper, dried at 60°C and stained with coommassie brilliant blue R-250. After staining for 1 hour, the plates were destained in methanol: acetic acid: water (5:5:1).

A standard reference human serum was used for construction of standard curve. The assay sensitivity was between 0.08 and 1.0 g apo B. Rocket area (rocket height x width at one half length) as a function of apoprotein concentration was measured to plot the standard curve. Fig. 5 shows the typical relationship between rocket height and apo B content of the reference serum.

ii) Apo E

The electroimmunoassay conditions for apo E were identical to those for apo B except that 116 g rabbit antihuman

apo E gammaglobulin per ml agarose was used. The operational range of the assay was between 0.02 to 0.20µg apo E (Fig. 5).

iii) Apo A-I

The electroimmunoassay of apo A-I employed 1.8% agarose, 0.06M sodium barbital buffer, (pH 8.6) with 2.5% dextran T-10 (Sigma) replacing the PEG. The other electroimmunoassay conditions were identical to those of apo B. The assay was used in the range of 0.02 to 0.2µg apo A-I (Fig 5).

iv) Apo C-II

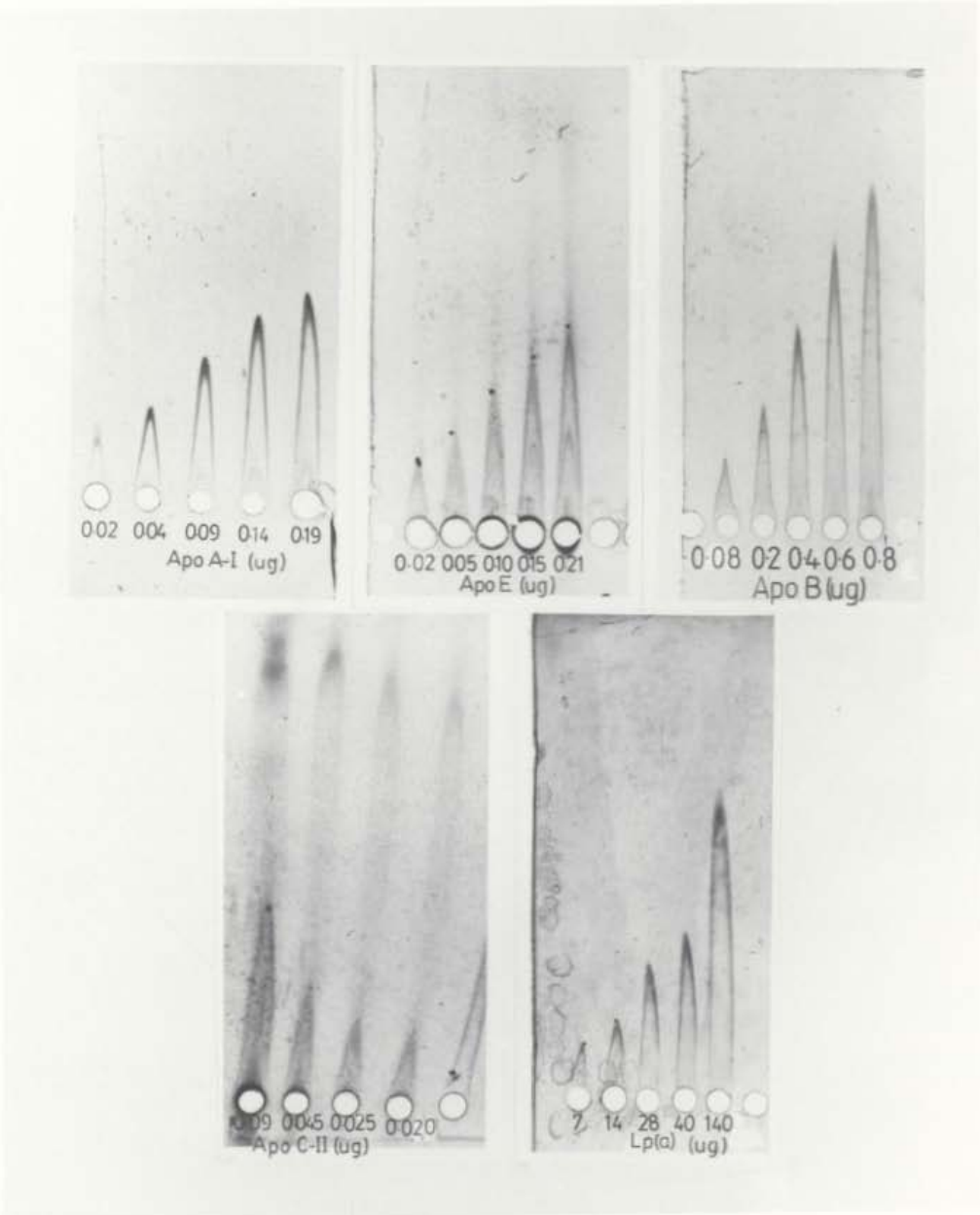
The electroimmunoassay of apo C-II was performed as described by Tam and Breckenridge (147). A 1.3% agarose gel with 2% PEG 6000 was prepared in 0.05M sodium barbital buffer (pH 8.8) containing 0.01% Triton X-100, 0.01 ml. BSA/ml gel and 75µg antibody per ml agarose. The electrophoresis was run at 2.5 V per cm/18h at 4°C. The plates were soaked successively in 0.15M NaCl for 1 hour and in distilled water for 15 min, covered with Whatman No. 1 filter paper, dried at 60°C and stained in coommassie brilliant blue R-250.

v) Lp (a)

For the electroimmunoassay of Lp(a) a 2% agarose gel was prepared in 0.06M sodium barbital buffer (pH 8.6) containing 0.4% PEG-6000. The electrophoresis was run at 2 V per cm for 21 h at 4°C. The assay was standardized with isolated Lp(a) which was a gift from Dr. W.C. Breckenridge, Department of Biochemistry, Dalhousie University.

Fig. 5. Electroimmunoassay of apoproteins.

The typical rockets obtained with standard reference serum (in case of Lp(a), isolated Lp(a) was used as reference) are shown. The validation and conditions for apo B, E and A-1 have been described previously by Dolphin et al. (146). The electroimmunoassay for apo C-II was performed following the conditions described by Tam and Breckenridge (147). All the assays showed a linear relationship between the peak area of the rocket and the apoprotein content of standard reference serum.


0·02 004 009 014 019
Apo A-I (ug)
0·02 005 010 015 021
Apo E (ug)
0·08 02 04 06 0·8
Apo B (ug)
0·09 0045 0025 0020
Apo C-II (ug)
2 14 28 40 140
Lp(a) (ug)

E. Analysis of apoproteins by high performance liquid chromatography (HPLC)

The separation of apoproteins of isolated VLDL and from the plasma fractions bound to Sepharose-PCBP was performed on a Perkin-Elmer, Series 4, HPLC System. Separation was performed on a TSK-250 gel filteration column (300x7.5 mM, Bio-Rad). The samples (containing about 100µg protein) were delipidated with ethanol:ether (3:1) at -20°C overnight. The apoproteins were dissolved in 100µl of 0.2 M Tris-HCl (pH 6.8) containing 3.6% SDS and 10% 2-mercaptoethanol. The sample solution was filtering through 0.45µM filters. An 50µl aliquot containing about 50µg protein was used for each chromatographic injection. The column was eluted with 0.5M sodium sulfate, 0.2M sodium phosphate buffer (pH 6.8) containing 0.1% SDS. The eluant was monitored at 280 nm at absorbance unit full scale (AUFS) of 0.32. The column was run at a flow rate of 1 ml per min.

F. Lipid Analysis

The lipids in the lipoprotein and plasma samples were quantitated by gas chromatographic (GC) total lipid profiling method (148). Total lipids (50-300µg) were first extracted by chloroform/methanol (2:1 v/v) following the method of Folch et al (149). An internal standard, tridecanoin, was added prior to the lipid extraction. Phospholipase C was added (for digestion, by vigorous agitation at 30°C for 2 hr) to aliquots of lipid samples along with Tris-HCl buffer

(pH 7.0), $CaCl_2.2H_2O$ (10% w/v) and ethyl ether (1 ml). The reaction was then stopped by addition of 0.1N HCl (5 drops). The organic phase was evaporated and the lipid residue treated with TRISIL-BSA (Pierce) and stored at -20°C until GC analysis. The lipid profiles were analyzed on an automated Hewlett Packard 5440 Gas Chromatograph using nickel columns (1/8x20 in) packed with 3% OV-1 on Gas chrom Q. The columns were programmed from 170 to 350°C. Response factors were established for each of the lipid components by analysis of standards. In some cases serum triglyceride and total cholesterol were estimated enzymatically on a Hitachi 705 Random axis analyzer using the Boehringer Mannheim automated analysis system. Total cholesterol was measured by the cholesterol esterase/cholesterol oxidase technique using commercial kit (CHOD-PAP test kit from Boehringer). The triglycerides were measured by the glycerol 3-phosphate-oxidase method (Boehringer).

G. Chemical modification of LDL

The lysine residues of LDL were modified by acetoacetylation using freshly distilled diketene (Sigma) as described by Weisgraber et al. (27). Diketene (either 1.29 or 4.0µmol) was added to LDL (1 mg protein) and the reaction allowed to proceed for 5 min at 25°C. The reaction was stopped by dialysis against 0.2M sodium carbonate/sodium bicarbonate buffer (pH 9.5). Control LDL was treated in similar fashion except that diketene was omitted.

1,2-cyclohexanedione (Aldrich) was used to modify selectively the arginine residues as described by Mahley et al (26). LDL (2-3 mg protein) was mixed with 0.15M 1,2 cyclohexanedione and incubated either for 2 or 3 h at 35°C. The sample was dialyzed for 40 h against 0.15M NaCl at 4°C. For control LDL, 1,2-cylohexanedione was excluded from the incubation mixture. The extent of lysine and arginine modification was determined by amino acid analysis as described below.

H. Amino acid analysis

Amino acid analysis of the various LDL samples was performed exactly as described by (26,27). Hydrolysis of samples (0.5 mg protein) was performed in 1 ml 6N HCl at 110°C for 24h in sealed tubes flushed with nitrogen and evacuated. The analysis was performed on Beckman Model 121-MB amino acid analyzer. Values reported were averaged from duplicate samples.

2.6. Assays and binding studies

A) Lipoprotein-heparin-Ca^{2+} precipitation assays

i) Assays with serum

The assays were performed essentially as described by Mookerjea (130). Serum from normal and inflamed rats and rabbits, male and female hamsters, DES-treated male hamsters and testosterone-treated female hamsters were used for the heparin-lipoprotein-Ca^{2+} precipitation assays. Serum from animals depleted of their PCPB, CRP and FP, as required, was

also used in the assays. The depleted serum was concentrated back to its original volume in a Amicon ultracentrifugation cell using a UM-10 membrane at 4°C before use. The standard assay mixture contained 50 mM Tris-HCl buffer (pH 7.6), heparin (50 units) $CaCl_2.2H_2O$ (concentration as specified for individual assays) P-choline (added at concentrations indicated for individual assays) and the respective serum (150µl) in a total volume of 2.5 ml. The assay was initiated by addition of the serum. In experiments where the effect of a particular protein was to be tested, the protein was also included in the incubation mixture. Incubation was carried out at 40°C for 1 hour with shaking. The turbidity developed as a result of lipoprotein precipitation was measured at 600 nm in a Pye-unicam spectrophotometer. Results reported are representative of at least three assays. Variations between the assays was less than 10%.

ii) Assays using purified human plasma lipoproteins

The assays were performed exactly as described above except that purified human plasma lipoproteins were used instead of whole serum. The protein concentration of VLDL or LDL used were 2.2 mg/ml and 2.6 mg/ml respectively.

B. Binding of ^{125}I-PCBP to liposomes

Assay mixtures to study the binding of ^{125}I-PCBP to liposomes contained 15 mM Tris-HCl buffer (pH 7.8), ^{125}I-PCBP (0.9 g), specified amounts of PCBP, BSA (0.1% w/v), liposomes of specified composition (600 nmols phospholipid), either in the presence or absence of

various concentrations of Ca^{2+} in a total volume of 0.5 ml. Specified concentrations of P-choline was included in the incubation mixture to examine its effect on the binding. Incubation was carried out in eppendorf centrifuge tubes at 37°C for 1 hour with shaking. The bound and unbound PCBP were separated by centrifugation at 12,800xg for 30 min. The amount of PCBP bound was calculated by counting the ^{125}I-PCBP in the sedimented liposomes using a Beckman 5500 gamma counter. The efficiency of liposome sedimentation was monitored by phospholipid phosphate analysis of the sediments (150,151). Each experiment was performed at least three times to compensate for variation in liposome preparation and representative values were used for the results. The variation between each assay was less than 10%.

C. Binding of lipoproteins to Sepharose-PCBP

Sepharose-PCBP columns (0.7x15 cm) were used to study the binding of human plasma and rat serum lipoproteins. The columns were equilibrated with 0.01M Tris-HCl buffer (pH 7.4) containing 0.15M NaCl and 10 mM Ca^{2+}. Plasma or isolated lipoproteins were brought to 10 mM with respect to Ca^{2+} concentration to study the binding in the presence of Ca^{2+}. After the application of the sample, the column was washed with the same buffer until absorbance at 280 nm of the eluant was less than 0.02. The columns were run at a flow rate of 20 ml per hour at 26°C and 2 ml fractions collected. The unbound fractions were collected and pooled. The bound

lipoproteins were eluted using a 0-250 mM P-choline gradient in the equilibration buffer. Further elution with 1.4M NaCl did not elute any additional lipoproteins. Sepharose-PCBP columns used for lipoprotein binding studies were regenerated after one use in the following way: The columns were washed consecutively with 4 bed volumes of 0.1M Tris-HCl buffer (pH 8.5) containing 0.5M NaCl and 0.1M CH_3COO Na buffer (pH 4.5) containing 0.5M NaCl and 10 mM EDTA. Finally the column was washed extensively with 0.01M Tris-HCl buffer, (pH 7.4) containing 0.15M NaCl.

D. Studies on the effect of PCBP on the binding of LDL to rat liver membrane receptors

i) Binding of ^{125}I-LDL to rat liver membranes

The binding assays were carried out essentially as described by Kovanen et al. (137). On the day of the experiment, the membrane (100,000xg pellets) preparation was thawed and resuspended in 1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and 1 mM Ca^{2+} by flushing ten times through a 22-gauge needle. The suspension was sonicated at 0°C for two 10 sec bursts in a sonifier cell disrupter (Model W185, Heat systems Ultrasonic Inc), using a microprobe at setting no. 6. The protein concentration of each suspension was adjusted with the same buffer to 10 mg/ml. The binding assay was performed by incubating the liver membranes (100-120 µg protein), specified amount of ^{125}I-LDL, buffer A (20 mM Tris HCl (pH 7.5) containing 20 mM NaCl and 20 mg/ml of bovine

serum albumin, to a final volume of 150 µl. The buffer contained either Ca^{2+} (5 mM) or EDTA (20 mM). Different concentrations of PCBP were included in the assay to test its effect on binding. Incubations were carried out in ice bath using Beckman microfuge tubes (1.5 ml capacity) at 0°C for 90 min with shaking. After incubation, 75µl of assay mixture was layered onto 200µl of fetal calf serum and centrifuged in a Beckman Type 25 rotor at 92,000xg for 30 min at 4°C. The supernatant was aspirated and replaced with 150 µl of fetal calf serum and the centrifugation repeated for 10 min. The supernatant was then removed and the whole tube assayed for radioactivity in a Beckman 5500 gamma counter. Control assays containing iodinated lipoproteins and PCBP were incubated exactly as described above except that these assays were carried out without any membranes. In these assays the radioactivity associated with the assays tubes was found to be less than 1% of the total radioactivity. The results of the binding assays and gel filtration studies (described below) are from a typical one of three separate experiments. The results in each case were reproducible. Each data point is the average of duplicate incubations which differed by less than 10%.

ii) Preincubation of liver membranes with PCBP.

Preincubation of liver membranes (120 µg protein) with increasing concentrations of PCBP in 'buffer A' was carried out for 90 min. at 0°C a total volume of 150µl. The

tubes were centrifuged at 12,800xg for 30 min. at 4°C, which resulted in quantitative sedimentation of the membranes as determined by the quantitative recovery of membrane protein in sediments. The supernatant was aspirated and replaced with ^{125}I-LDL (48 µg protein/ml) and 'buffer A'. The membranes were gently resuspended and incubated for an additional 90 min at 0°C, and then centrifuged as described for the liver membrane binding assays. The amount of ^{125}I-LDL bound to the liver membrane was determined from the radioactivity associated with the membrane sediments. The results presented are average of duplicate assays. In a separate experiment, liver membranes (120µg) were incubated in the presence of 1µg of ^{125}I-PCBP plus unlabelled PCBP to give indicated concentrations, and 'buffer A' for 90 min at 0°C, in a final volume of 150µl. The incubation mixture was centrifuged at 12,800xg for 30 min and the supernatants discarded. The membrane sediments were washed (twice) with 'buffer A' and counted for radioactivity to determine the amount of ^{125}I-PCBP bound.

ii) Gel-filtration experiments

LDL (0.6 mg protein) and ^{125}I-PCBP (0.5µg ^{125}I-PCBP diluted with 36.5µg unlabelled PCBP) were incubated in 0.01M Tris-HCl buffer (pH 7.4) containing 100 mM NaCl either in the absence or presence of Ca^{2+} (5 mM) in a total volume of 0.2 ml at 0°C for 90 min and the mixture applied to a Sephacryl

S400 column (1.5 x 45 cm). The column was eluted with 0.01M Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, and Ca^{2+} (5 mM) at a flow rate of 24 ml/h. The fractions (2 ml) were counted for radioactivity in a Beckman 5500 gamma counter. The elution volumes for LDL and ^{125}I-PCBP were determined in separate experiments. The void volume of the column was determined using Blue Dextran-2000.

In another set of experiments, the supernatants (100µl) from the LDL receptor binding assays carried out in buffer A (containing 2 mg/ml BSA instead of 20 mg/ml) were chromatographed on Sephacryl S400 column as described above. The supernatants were obtained by centrifugation of the assay mixture at 12,800xg for 30 min at 4°C.

2.7. Plasmapheresis experiments

A. - In vitro studies

Sepharose-PCBP, for these studies, was prepared exactly as described before to give a concentration of 1 mg PCBP per ml of Sepharose. To study the binding of lipoproteins from plasma, Sepharose-PCBP columns (2 ml bed volume; 0.5x10 cm) were equillbrated with 0.01M Tris-HCl buffer containing 0.15M NaCl, (pH 7.4). In experiments where the effect of Ca^{2+} concentration on the binding of lipoproteins from plasma was to be studied, the equilibration buffer contained the desired amount of Ca^{2+}. The plasma sample before application to the column was brought to the desired Ca^{2+} concentration.

To study the effect of the amount of cholesterol in the

plasma on the binding of plasma lipoproteins, increasing volumes of hypercholesterolemic plasma were applied to Sepharose-PCBP. In all the in vitro experiments the columns were operated at a flow rate of 20 ml/h at 25°C and 0.5 ml fractions were collected. The eluant was continously monitored at 280 nm and the columns were eluted until no further material absorbing at 280 nm was eluted. The bound fractions were eluted by 20 mM EDTA solution. Subsequent to this elution passage of either 100 mM P-choline or 1.4M NaCl did not elute any additional lipoproteins thus indicating that the elution by EDTA was quantitative.

The total cholesterol and triglyceride content of plasma as well as the plasma fraction bound to Sepharose-PCBP columns were estimated by enzymatic method as described before. HDL-cholesterol and VLDL + LDL-cholesterol content of the plasma samples and bound fractions was measured after the precipitation of VLDL + LDL fraction using a heparin/manganese precipitation procedure (152). By this procedure the apo B containing lipoproteins, VLDL and LDL, are precipitated, leaving HDL and its associated cholesterol in the supernatant. The difference between total cholesterol content and the HDL cholesterol gives the VLDL + LDL cholesterol values. The heparin/manganese precipitation assay was performed as follows:

To each ml of plasma or the bound fraction, 40 µl of heparin solution (5000 units/ml) and 50µl of 2 M $MnCl_2$ solution

were added, followed by thorough mixing after each addition. The samples were incubated for 10 min at 25°C, then centrifuged for 30 min at 1500xg at 4°C. The supernatants were quickly transferred and stored at 4°C until cholesterol estimation by the enzymatic method described before. Protein content of the fractions bound to Sepharose-PCBP were estimated by the modified method of Lowry et al. (141).

B. In Vivo Studies

i) Surgical Procedures

a) Anesthesia: The rabbit was anesthetized by an intramuscular injection consisting of the anesthetic ketamine at a dose of 35 mg/kg body weight combined with the sedative/analgesic xylazine (Rompum) at 5 mg/kg body weight.

After the animal had attained the surgical plane of anesthesia, the neck region, back region and the areas over the right and left femoral arteries were shaved. The rabbit was then transferred to the operating table and positioned within a cradle restrainer in a supine position. A heating pad and a grounding plate were placed under the rabbit. Each limb was taped securely to the cradle to maintain the proper position.

Halothane, nitrous oxide and oxygen were administered to the animal in concentrations of 1%, 0.4L and 2L respectively. The respirator was set at a rate of 35 breaths per minute with a tidal volume of 250 ml/kg. Prior to tracheostomy, this mixture was administered via a respirator mask. Once

the tracheostomy was performed, the respirator was hooked up to the tracheal tube.

b) Tracheostomy and Vessel Isolation

A ventral midline incision was made in the neck through the skin and subcutaneous tissue using electrocautery. Any blood vessels transected by the incision were cauterized. The midline of the exposed muscle layer was found and blunt dissected using Metzanbaum scissors. The trachea was thus exposed.

An incision was made in the trachea using a #22 blade fitted on a #4 scalpel handle. A tracheostomy tube of appropriate size was inserted. Moistened umbilical tape was passed around the trachea and tube, and used to tie the tube in place. As stated previously, the respirator was then hooked up to the tube.

The carotid artery and jugular vein were isolated by blunt dissection through the fascia. A piece of 4-0 mersilene was passed around each vessel and clamped with mosquito forceps. The neck incision was then covered with moistened gauze.

(c) Femoral Artery Isolation: An oblique ventral incision was made in the right hindleg of the rabbit. Blunt dissection was used to isolate the femoral artery. Approximately 3 to 4 cm of the artery was exposed.

Two pieces of 4-0 mersilene were passed around the vessel and the vessel was ligated distally. A small nick was

made in the vessel with Potts Smith cardiovascular scissors and a PE-90 cannula was inserted. The second piece of mersilene was tied securely around the cannula and vessel such that the cannula would not slip. The external end of the cannula was attached to an arterial pressure transducer. The incision was covered with moistened gauze.

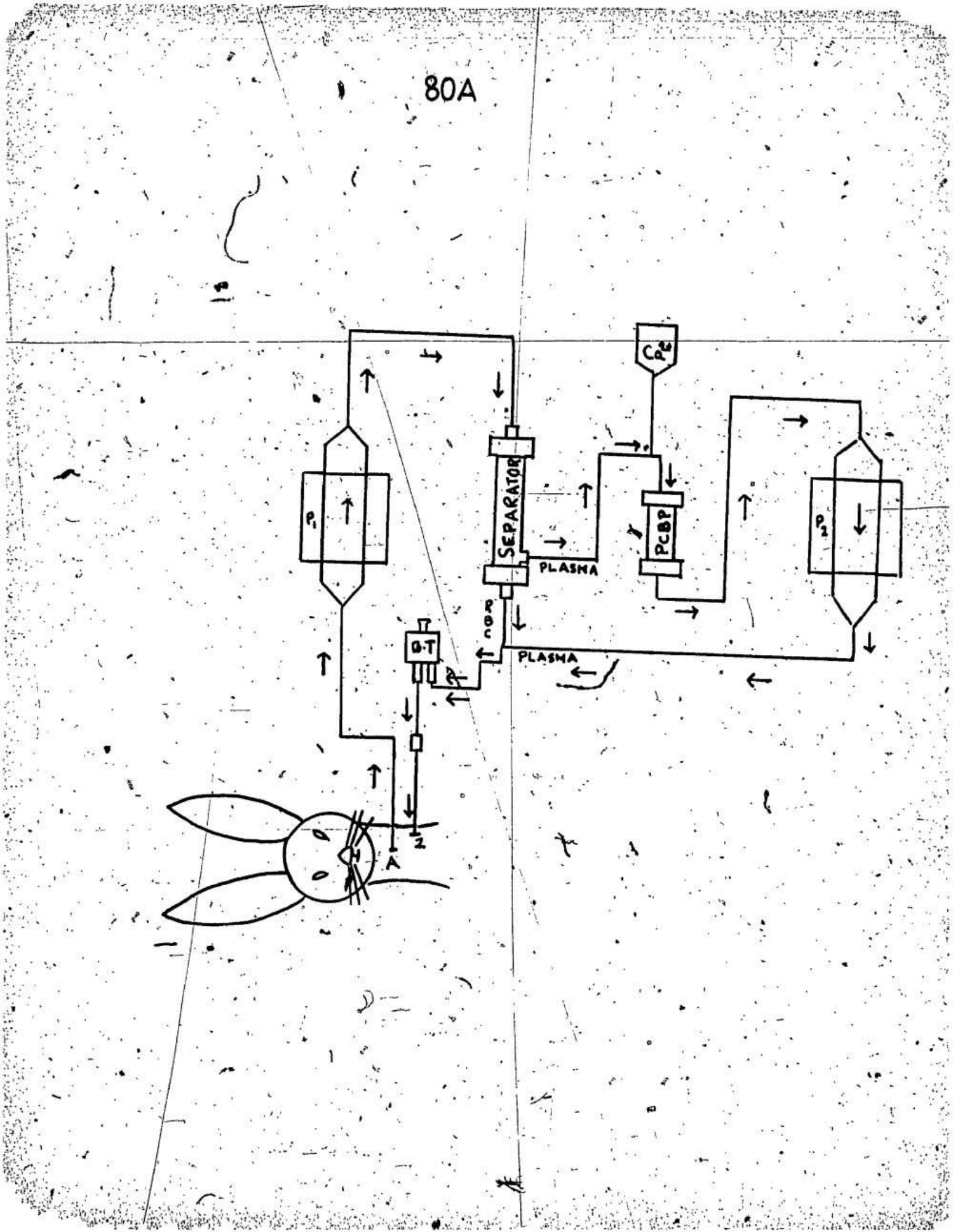
(d) Cannulation of the Jugular Vein and Carotid Artery: The jugular vein (previously isolated) was ligated cranially. A couple of drops of papaverin were placed on the exposed vessel to promote vessel dilation. A small nick was made in the vessel and a size 8 French catheter was inserted. A piece of 4-0 mersilene was used to secure the cannula in place. The external end of the catheter was attached to an IV set which was in turn connected to the bubble trap as per Figure 6.

Similarly, the carotid artery was ligated cranially. A small nick was made in the vessel and a PE-190 cannula inserted. Mersilene (4.0) was used to secure the cannula in place. The external end of the cannula was attached to the tubing going through P_1 as per Figure 6. The incision area in the neck was covered with moistened gauze.

The animal was heparinized with 300 iu/kg body weight heparin and subsequently heparinized every hour with 150 iu/kg body weight. The temperature of the rabbit was monitored through the use of a rectal electrotherm thermometer. Blood samples for hematocrit determinations were taken from venous

Fig. 6. Illustration of the experimental extracorporeal circuit for plasmapheresis.

Blood was withdrawn from the carotid artery (A) and run through peristaltic pump (P_1) to a plasma separator. The plasma was separated from the red blood cells (RBC) and was channelled into a Sepharose-PCBP column. $CaCl_2.2H_20$ (5 mM) was dripped from a reservoir into the plasma before it entered the column. After flowing through the column, the plasma was returned to mix with the RBC's with the help of peristaltic pump (P_2). The mixture was passed through a pediatric bubble trap (B.T.) and the blood returned to the jugular vein (Z). The arrows indicate the direction of blood/plasma.


P_1
SEPARATOR
PLASMA
C_{P_2}
PCBP
P_2
RBC
PLASMA
B.T
A
H
2

catheter.

ii) Plasmapheresis system

The carotid artery cannula was attached to silastic tubing (OD - 5/16, ID = 3/16) which was run through a peristaltic pump (P_1) to a Travenol plasma separator (Fig. 6). Blood was drawn from the artery at a rate of 22 ml per min. The plasma was separated from the RBC's and was channelled into a Sepharose-PCBP column. $CaCl_2.2H_2O$ (5 mM solution) was dripped into the plasma before it entered the column at a rate of 1 ml per minute into the plasma at stopcock A through the length of the experiment. After flowing through the Sepharose-PCBP column, the plasma was returned, to mix with the red blood cells, with the help of peristaltic pump P_2. The mixture was passed through a Pediatric Bubble Trap and the blood was returned to the jugular vein, at a rate of 5 ml per min.

Prior to experimentation, the system was primed with Lactated Ringers solution (180 ml) containing 0.2% heparin 2% rheomacodex and adjusted to a pH of 7.7. Arterial blood samples were monitored before passing through the plasma separator and venous blood samples just prior to reinfusion into the animal to determine pre- and post-plasmapheresis concentrations of albumin and total protein. To determine the amount of lipoprotein bound to Sepharose-PCBP during plasmapheresis, the column was washed exhaustively with 0.01M Tris buffer (pH 7.4) containing 0.15M NaCl and 2.5 mM

Ca^{2+} till the absorbance at 280 nm of the eluant was below 0.02. The bound lipoprotein was then eluted with 20 mM EDTA and the eluate fractions analyzed for total cholesterol, VLDL + LDL cholesterol, HDL cholesterol, triglycerides, and total protein as described for the in vitro experiments.

CHAPTER 3

STUDIES ON THE EFFECT OF PCBP, FP, RABBIT AND HUMAN CRP ON HEPARIN-LIPOPROTEIN PRECIPITATION.

3.1. Introduction

PCBP was previously shown to inhibit heparin-serum[1] lipoprotein precipitation in the presence of Ca^{2+} (131). The results of this study suggested that PCBP due to its phosphorylcholine binding property, may interact with PC molecules exposed on the surface of the lipoproteins and thereby prevent the precipitation. This leads to the hypothesis that other phosphorylcholine binding pentraxins may also demonstrate similar inhibition of heparin-lipoprotein precipitation.

The purpose of the present investigation was to study the effects of PCBP, FP, rabbit and human CRP on heparin-lipoprotein precipitation. The effects of pentraxins on heparin-lipoprotein precipitation reaction have been studied using serum from animals with different treatments, ie, rats and rabbits with inflammation, male and female syrian hamsters treated with sex hormones. Animals treated as above are known to have increased or decreased levels of pentraxins in their serum (78,82,126). Studies were done

[1]Although the words 'serum lipoprotein' have been used as rather general terms through out the text in describing lipoprotein-heparin interactions, it is well recognized that only apo B containing lipoproteins (VLDL and LDL) form insoluble complexes with heparin in the presence of Ca^{2+} (55).

also by addition of purified pentraxins to the heparin-lipoprotein precipitation assays using different kinds of sera. In addition, studies were done using purified VLDL and LDL instead of whole serum as a source of lipoproteins to monitor the effect of pentraxins on heparin-lipoprotein precipitation reaction uncomplicated by the effects of other proteins present in whole sera.

Since the heparin-lipoprotein precipitation reaction is directly related to the concentration of lipids in the sera (55) the levels of triglyceride and total cholesterol in different sera used have been measured.

The role of sialic acid residues present on PCBP and FP in their effects on heparin-lipoprotein precipitation has been examined.

These studies may provide added insights into the nature and mechanism of the effect of PCBP and other pentraxins on heparin-lipoprotein precipitation reaction.

3.2. Results

A. Comparison of heparin-lipoprotein precipitation using serum from control and treated animals.

(i) Rat

The results (Table 5) show no significant difference in triglyceride and total cholesterol levels in inflamed serum compared with control serum, while PCBP levels increased by two fold. Heparin-lipoprotein precipitation reaction measured

Table 5

Comparison of lipid and phosphorylcholine binding pentraxin levels in serum of control and experimental animals

Animal	Triglyceride (mg/dL)	Total cholesterol (mg/dL)	PCBP (mg/ml)	CRP (mg/ml)	FP (mg/ml)
Rat					
Control	138±6.25	64±2.0	0.64±0.07	-	-
Inflamed	118±10.10	60±1.9	1.21±0.2.0 (P<0.00001)		
Rabbit					
Control	87±10.18	35±7.45		Trace	
Inflamed	176±26.28 (P<0.05)	71±8.92 (P<0.025)	-	0.11±02	-
Hamster					
Male	168;146	106;114			0.013;0.01
Female	131;129	105;108	-	-	1.00;1.20
DES treated Male	247±76.30	216±13.37			1.072±0.13
Testosterone treated female	267±28.04	151±9.77 (P<0.0025)*			0.058±0.013 (P<0.0001)*

Note: All values are expressed as the mean ± SE of the mean except the results of control male and female hamsters which represent the serum values from two animals. Serum from five animals was examined in each group for rats, DES treated male hamsters and testosterone treated female hamsters. Serum from eight animals was examined in each group for rabbits. Statistical significance was calculated with Students unpaired t-test.

*indicates statistically significant difference from DES treated male hamster serum.

Total cholesterol and triglyceride were estimated by enzymatic method using the Boehringer Manheim automated analysis system on a Hitathi 705 Random Axix Analyser. The concentrations of PCBP, CRP and FP were determined as described in Chapter 2.

-indicates not present

as a function of Ca^{2+} was lower with serum from inflamed rats compared to control serum (Fig. 7a and b). Addition of 16 µ M P-choline resulted in increased precipitation in the region of 10-30 mM Ca^{2+} in control serum, while addition of up to 32 µM P-choline was required to produce maximum increase in precipitation with serum from inflamed rats (Fig. 7a and b).

(ii) Rabbit

A large increase in triglyceride, total cholesterol and CRP levels was seen in serum from inflamed rabbits as compared to control serum (Table 5). Heparin-lipoprotein precipitation reaction was greatly increased in assays with serum from inflamed rabbits compared to control rabbit serum (Fig. 8a and b).

(iii) Hamster

The serum triglyceride and total cholesterol levels were not significantly different between male and female hamsters (Table 5), but as expected from the work of Coe (126), the level of FP was markedly higher in the female hamster (Table 5). A comparison of heparin-lipoprotein precipitation as a function of Ca^{2+} concentration showed lower precipitation reaction in assays with female serum than male serum (Fig. 9). However, depletion of FP from female serum resulted in increased precipitation (Fig. 9).

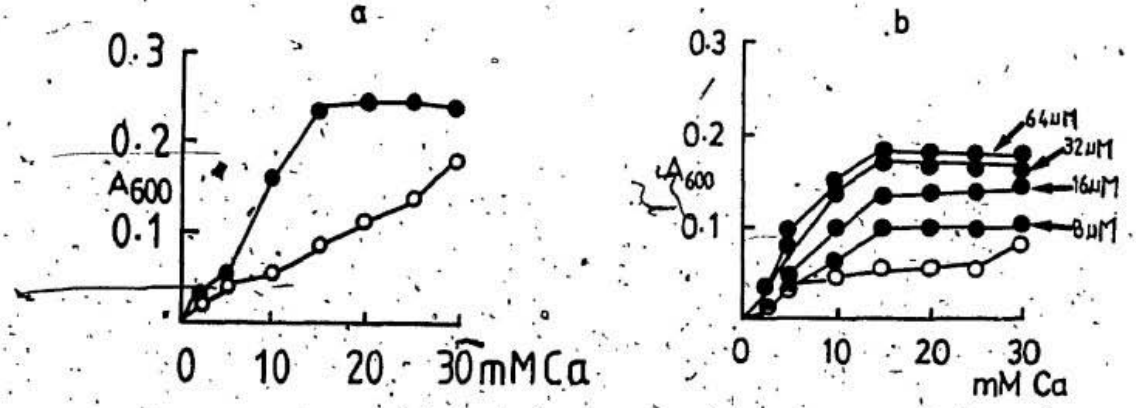


Fig. 7. Comparison of heparin-serum lipoprotein precipitation reaction in control and serum from inflamed rats as a function of Ca^{2+} concentration. Assays were carried out using 150 µl serum from (a) control rats (b) inflamed rats either in the absence or presence of 16 µM P-choline for (a) and other concentrations as indicated for (b). Heparin (50U) and Tris-HCl (50 mM; pH 7.6) were present in all assays in a final volume of 2.5 ml. The tubes were incubated with shaking at 40°C for 60 min and then mixed using a vortex mixer before reading the absorbance at 600 nm.

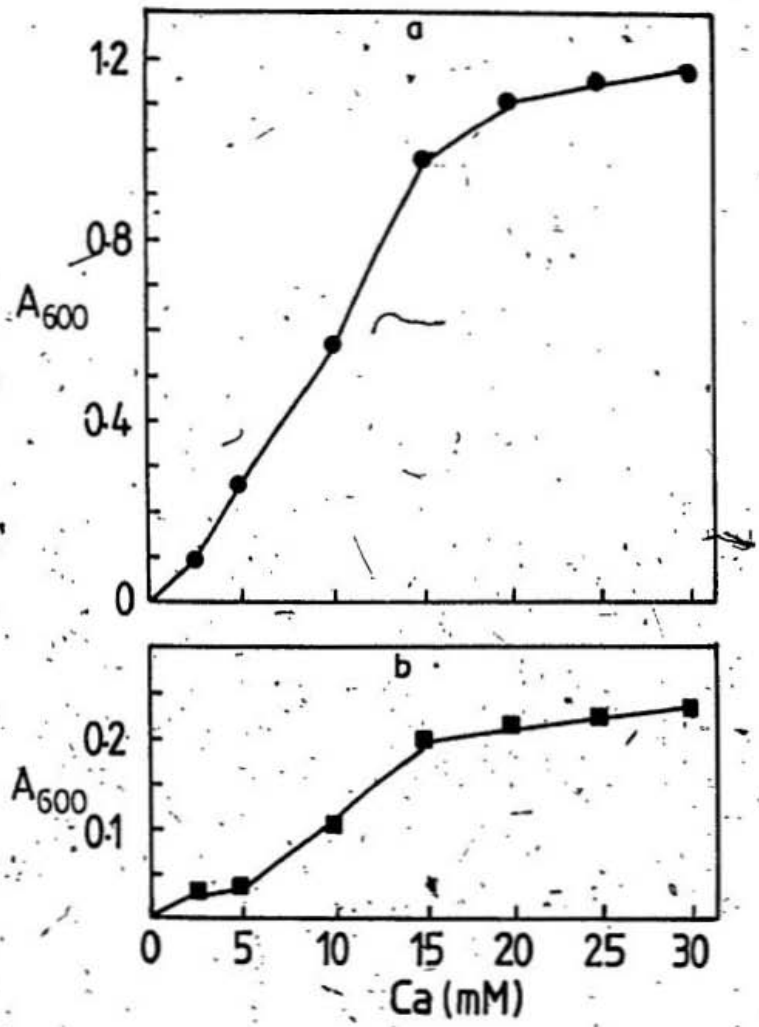


Fig. 8. Comparison of heparin-serum lipoprotein precipitation reaction in control rabbit serum and serum from inflamed rats as a function of Ca^{2+} concentration. Assays were performed using 150 µl of (a) Inflamed rabbit serum and (b) control rabbit serum (■). Other experimental details are as described in the legend to Fig. 7.

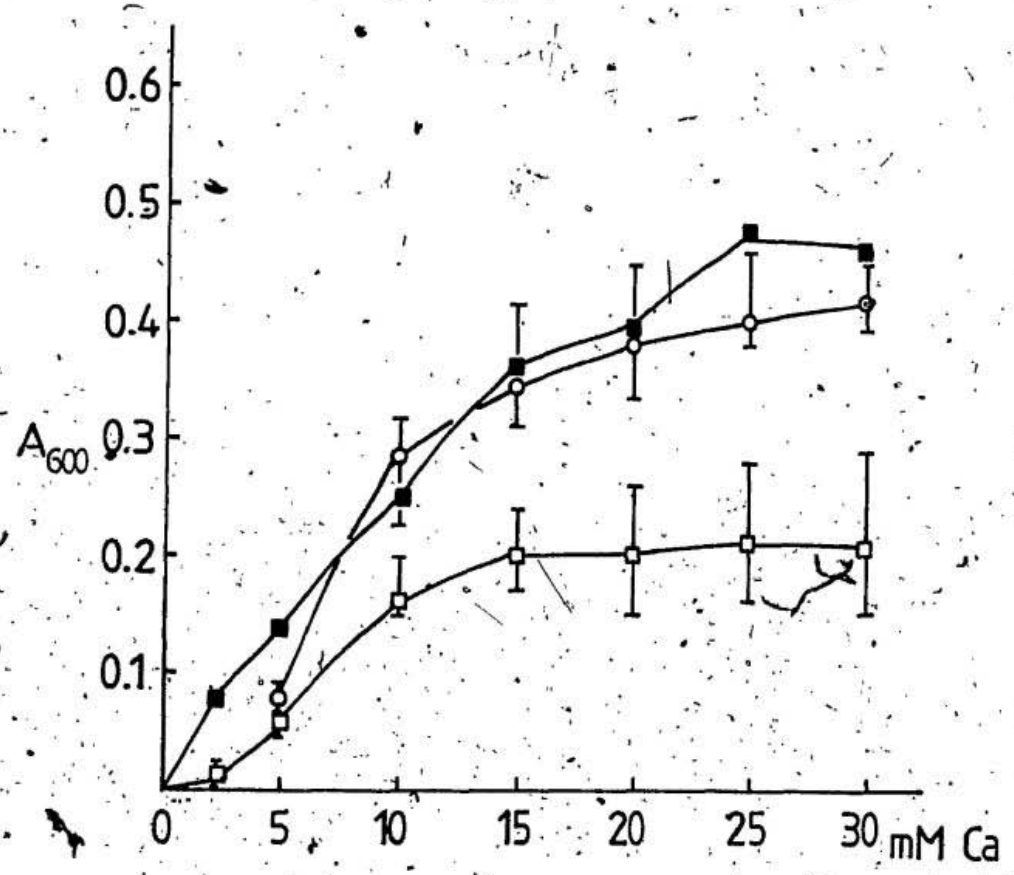


Fig. 9. Comparison of heparin-serum lipoprotein precipitation reaction in male, female and FP-depleted female hamster serum as a function of Ca^{2+} concentration. Assays were performed using male (O), female (-□-) and FP-depleted female hamster serum (-■-). 150 µl of serum was used in each case. Details of the assays are as described in legend to fig. 7. In case of male and female serum, results are presented as range of values (bars) and mean of values (symbols) where number of sera examined = 3.

The results with serum from DES and testosterone treated hamsters showed that the total cholesterol levels were lower in the testosterone treated female hamster serum compared to DES treated male hamster serum, while the triglyceride levels were higher in the former. Since FP is regulated by sex hormones (126), DES treatment in male hamsters induced FP (compare with control male hamster, Table 5) and testosterone treatment in female hamsters reduced FP levels (compare with control female hamsters Table 5). When heparin-lipoprotein precipitation was studied as a function of Ca^{2+}, the precipitation reaction was lower in DES treated male serum compared to testosterone treated female serum (Fig. 10).

B. Effect of purified pentraxins on heparin-serum lipoprotein precipitation.

(i) PCBP

PCBP was previously shown to have an inhibitory effect on heparin-lipoprotein precipitation reaction using PCBP-depleted rat serum (131). In the present study the inhibitory effect was also demonstrable when PCBP was added to assays using CRP depleted serum from inflamed rabbits and control rabbit serum. As shown in Fig. 11, addition of increasing amounts of PCBP inhibited the precipitation reaction using either serum.

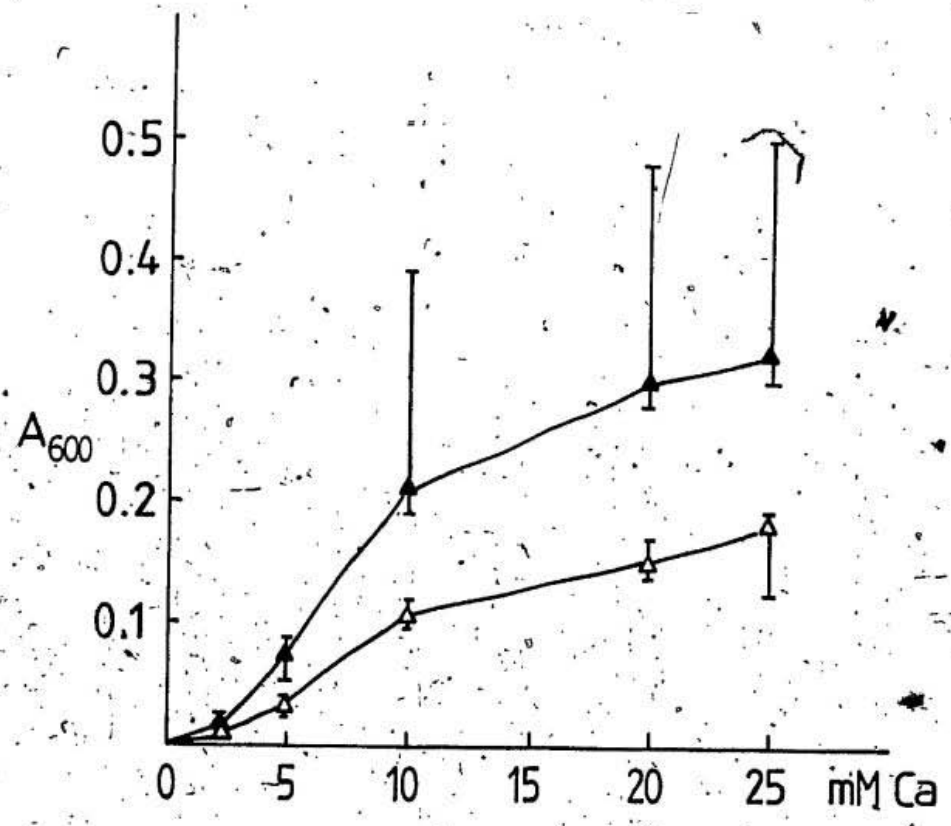


Fig. 10. Comparison of heparin-serum lipoprotein precipitation reaction in DES treated male hamster and testosterone treated female hamster serum as a function of Ca^{2+} concentration.

The assays were performed with 150 µl of DES treated male serum (Δ) and testosterone treated female serum (▲). Details of the assays are as described in the legend to fig. 7. Results are presented as range of values (bars) and mean of values (symbols) when number of sera examined = 3.

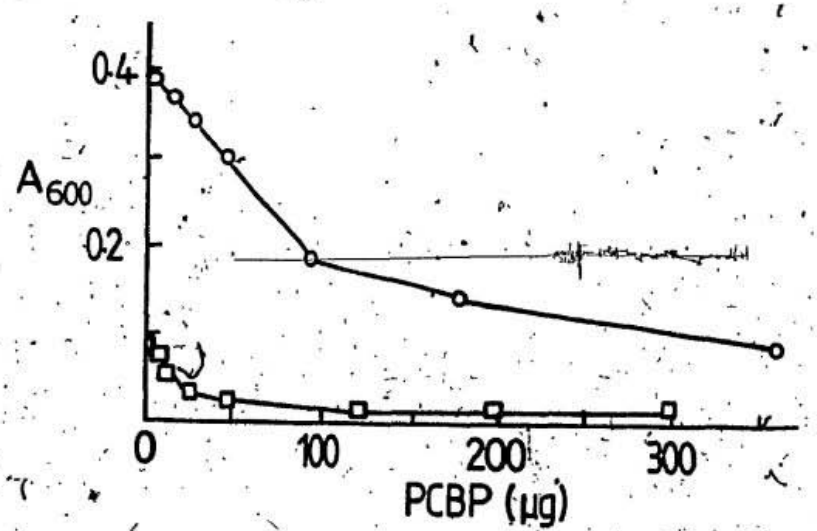


Fig. 11. Effect of addition of increasing amounts of PCBP on heparin-serum lipoprotein precipitation reaction.

Assays contained either 150 µl of CRP-depleted rabbit serum (O) or control rabbit serum (□) and were performed in presence of 10 mM Ca^{2+}. Other details of the assay are as described in legend to fig. 7.

(ii) Rabbit CRP

CRP isolated from inflamed serum was added in increasing amounts to assays using CRP-depleted rabbit serum and PCBP-depleted rat serum (Fig. 12). Addition of up to 300 µg of CRP showed no inhibitory effect on the serum lipoprotein precipitation reaction using either serum.

(iii) FP

The effect of addition of FP on serum lipoprotein precipitation reaction using male hamster or FP-depleted female hamster serum is shown in Fig. 13. Addition of increasing amounts of FP to assays with both sera resulted in inhibition of lipoprotein precipitation reaction.

C. Comparison of the effects of pentraxins on heparin-lipoprotein precipitation using human VLDL and LDL.

Pentraxins, viz. PCBP, FP, rabbit and human CRP were added individually in increasing amounts to assays using purified human VLDL or LDL (Fig. 14a and b). The addition of PCBP or FP resulted in inhibition of the precipitation reaction in assays with both lipoproteins. In contrast rabbit and human CRP showed no inhibitory effect on the precipitation assays using purified lipoproteins.

Human SAP and fetuin are sialic acid containing glycoproteins which do not bind to phosphorylcholine ligand (77). Addition of these proteins to assays using VLDL

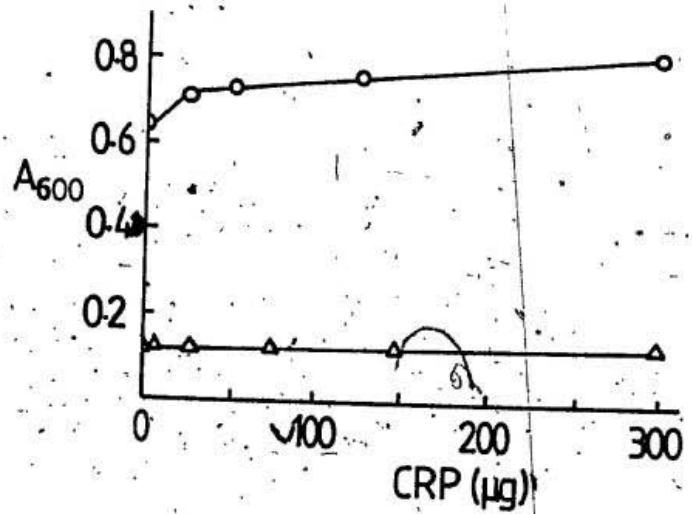


Fig. 12. Effect of addition of increasing amounts of rabbit CRP on heparin-serum lipoprotein precipitation reaction. The assay mixture contained either 150 µl of CRP-depleted serum from inflamed rabbits (O) or PCBP-depleted control rat serum (Δ) and were performed in the presence of 15 mM Ca^{2+}. Other experimental details are as described in legend to Fig. 7.

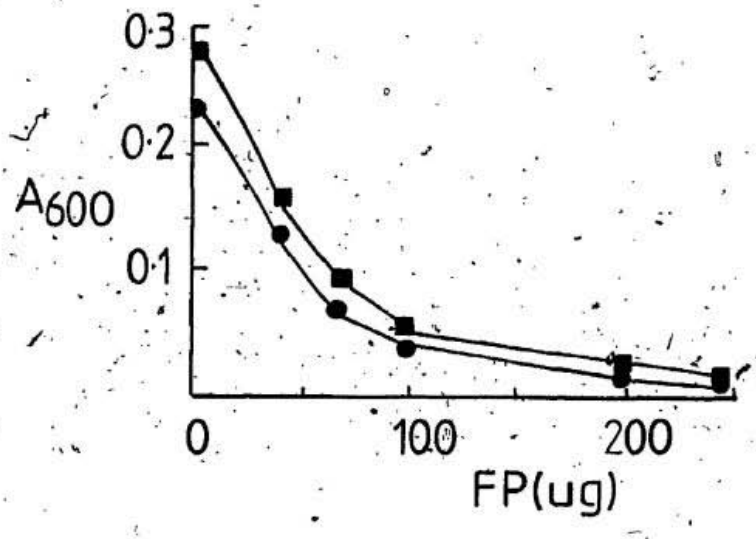


Fig. 13. Effect of addition of increasing amounts of FP on heparin-serum lipoprotein precipitation reaction. Assays contained 150 µl of either male hamster serum (■) or FP-depleted female hamster serum (●) and were performed at 10 mM Ca^{2+}. Other details of the assays are as described in legend to Fig. 7.

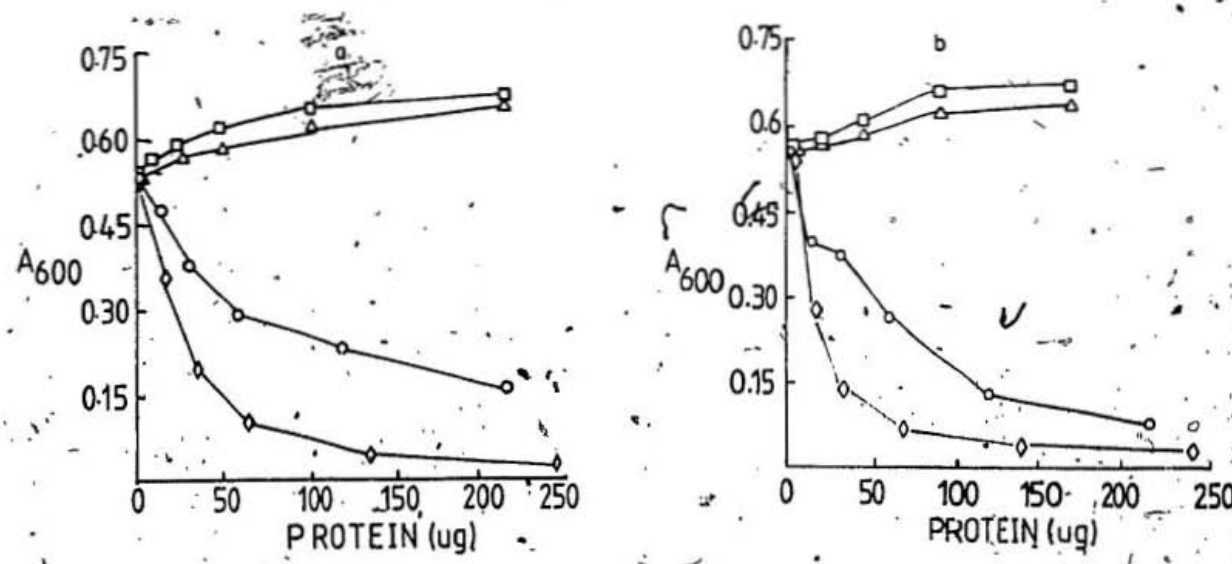


Fig. 14. Comparison of the effect of addition of increasing amounts of pentraxins on heparin-lipoprotein precipitation reaction. Assays contained increasing amounts of either one of the following pentraxins - PCBP (O), FP (◊), rabbit CRP (Δ), or human CRP (□) and were carried out with either a) human VLDL (100 µl; 220 µg protein) or b) human LDL (100 µl; 260 µg protein). All assays were performed at 10 mM Ca^{2+}. Other details are as described in legend to Fig. 7.

showed no inhibitory effect on the heparin-VLDL precipitation reaction (Fig. 15).

D. Comparison of the effect of desialylated pentraxins with native pentraxins on heparin-lipoprotein precipitation.

(i) PCBP

Treatment of PCBP with neuraminidase enzyme led to 93% depletion of sialic acid (Table 6). The desialylated PCBP showed reduced mobility on PAGE (139) when compared with PCBP (Fig. 16). Desialylated PCBP bound to Sepharose-phenylphosphoryl-choline affinity adsorbent could be eluted at an identical concentration (7.25 mM) of phosphorylcholine required for the elution of PCBP (Fig. 17). The desialylated PCBP cross reacted against antiserum to PCBP and maintained a continuous line of immunochemical identity with PCBP (Fig. 18). The CD spectra of desialylated PCBP in the aromatic and UV region of the spectrum was essentially the same as that of (Fig. 19a and b). These results suggested that no detectable denaturation had occurred on desialylation. However when desialylated PCBP was added (up to 500µg) to heparin lipoprotein precipitation assays with human VLDL, it showed, no inhibitory effect on the precipitation reaction (Fig. 20). Free sialic acid in the presence or absence of desialylated PCBP had no effect on heparin-VLDL precipitation reaction (Fig. 21).

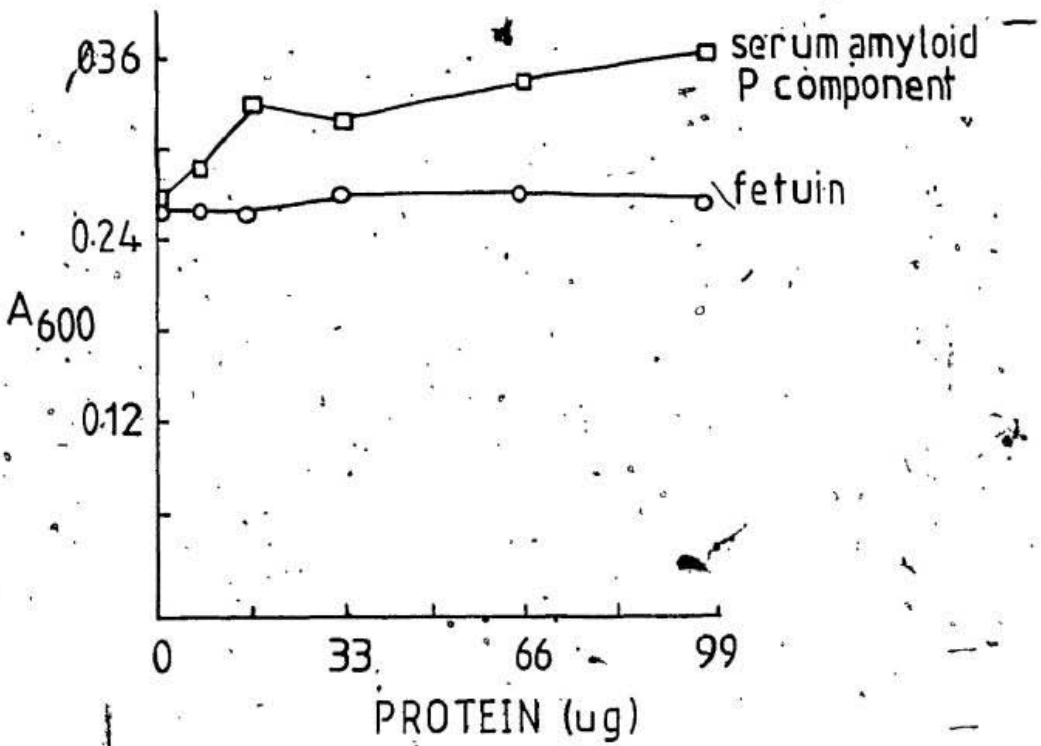


Fig. 15. Effect of addition of increasing amounts of human SAP and fetuin on heparin-VLDL precipitation reaction. Assays were carried out using VLDL (50 µl; 110 µg protein) in the presence of increasing amounts of either human SAP (□) or fetuin (O) and were performed using 10 mM Ca^{2+}. Other details of the assay are as described in legend to Fig. 7.

Table 6

Desialylation of PCBP and FP by neuraminidase treatment

Amount of sialic acid present	Amount of sialic acid released by neuraminidase	% Removal of sialic acid
PCBP		
10.6 mol/mol protein	9.66 mol/mol	91
FP		
7.4 mol/mol protein	2.86 mol/mol	38.6

Desialylation of both pentraxins was performed enzymatically by neuraminase treatment. PCBP (10 mg) or FP (3 mg) were incubated in 5 mM Tris-HCl buffer (pH 7.6) with 2.5 Units and 1 Unit of neuraminidase respectively at 37°C for 12h. Release of sialic acid was followed using Warren's method (140) for sialic acid estimation. Other details of the incubation are described in Chapter 2.

Fig. 16. PAGE of purified PCBP, desialylated-PCBP and a mixture of PCBP and desialylated-PCBP.

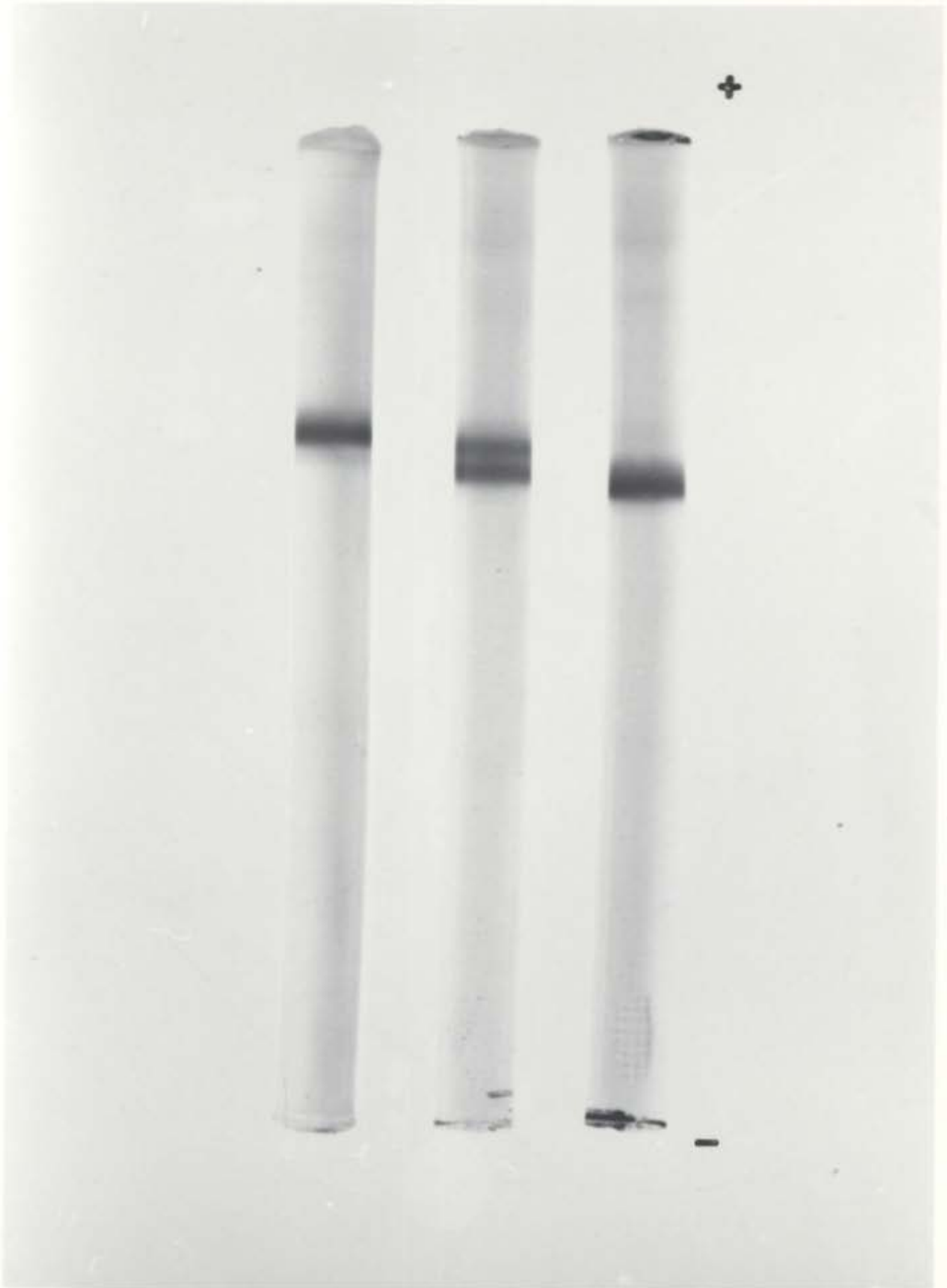
PAGE was performed on 7% polyacrylamide gels according to the method of Davis (139).

Gel A: desialylated-PCBP (10 µg).

Gel B: mixture of PCBP and desialylated-PCBP (10 µg each).

Gel C: PCBP (12 µg).

100A



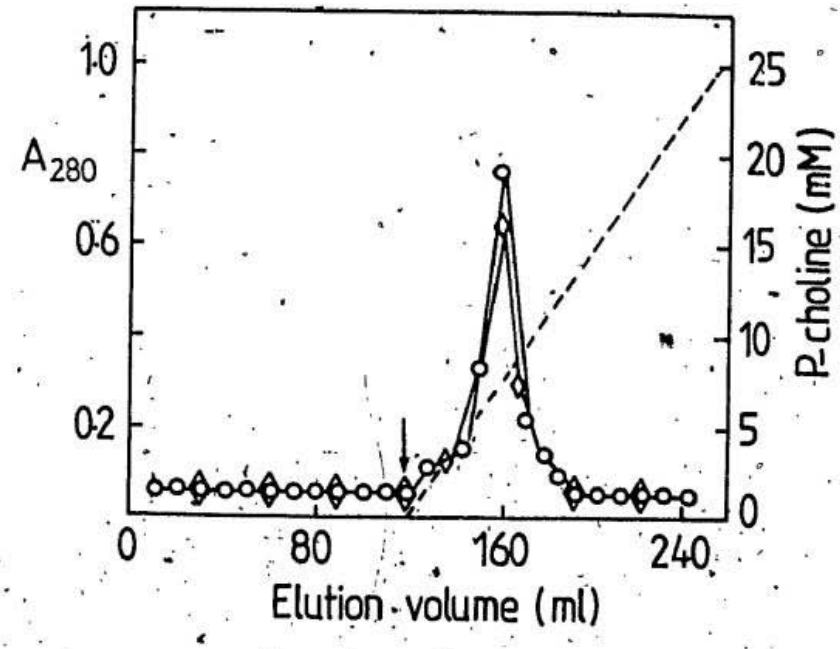


Fig. 17. Gradient elution of PCBP and desialylated PCBP bound to Sepharose-phenylphosphorylcholine affinity adsorbent by P-choline.

PCBP and desiaylated PCBP were applied separately to Sepharose-phenylphosphorylcholine columns (30 ml bed volume). The bound proteins were eluted at 7.25 mM P-choline upon application of a P-choline gradient (0-25 mM). The recovery for both proteins was 94%.

Fig. 18. Ouchterlony double diffusion analysis of the desialylated PCBP using antiserum to PCBP.

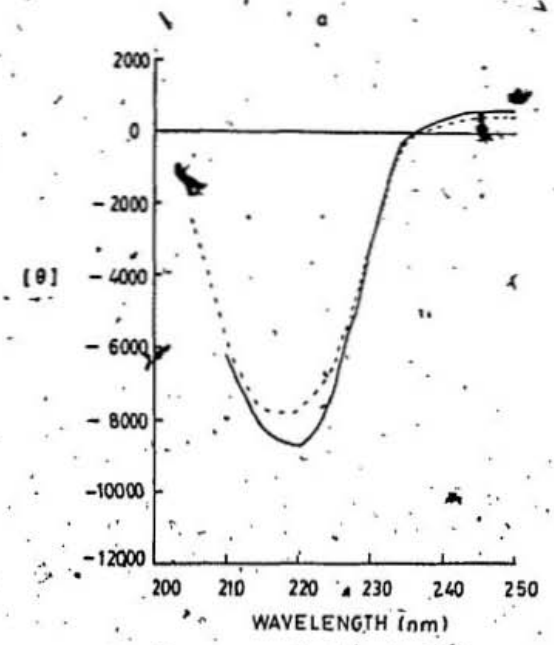
Center well has antiserum to PCBP raised in rabbits.

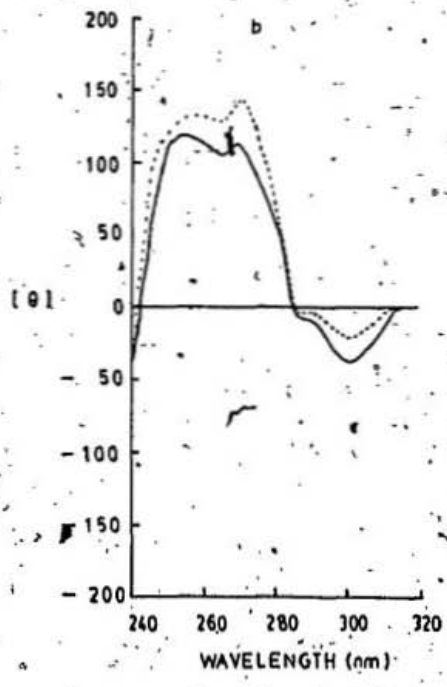
Wells #2,4 and 5 contain purified PCBP.

Wells #1 and 3 contain purified desialylated-PCBP.

Fig. 19. Circular dichroism spectral analysis in the far-UV and aromatic system of PCBP and desialylated PCBP.

(a) Circular dichroism spectral analysis in the far-UV region of PCBP and desialylated PCBP. PCBP (——) 0.5 mg/ml in 5 mM Tris-HCl buffer (pH 7.6) and desialylated PCBP (----) (0.48 mg/ml in 5 mM Tris-HCl buffer (pH 7.6) were used to perform the spectral analysis. Experiments were performed at 25°C (cell length = 0.1 cm). (b) Circular dichroism spectral analysis of PCBP and desialylated PCBP in the aromatic region. Experiments were performed under exactly similar conditions as mentioned in (a) except that the cell length was 2 cm.


a
2000
0
-2000
-4000
-6000
-8000
-10000
-12000
[θ]
200
210
220
230
240
250
WAVELENGTH (nm)


b
200
150
100
50
0
-50
-100
-150
-200
[θ]
240
260
280
300
320
WAVELENGTH (nm)

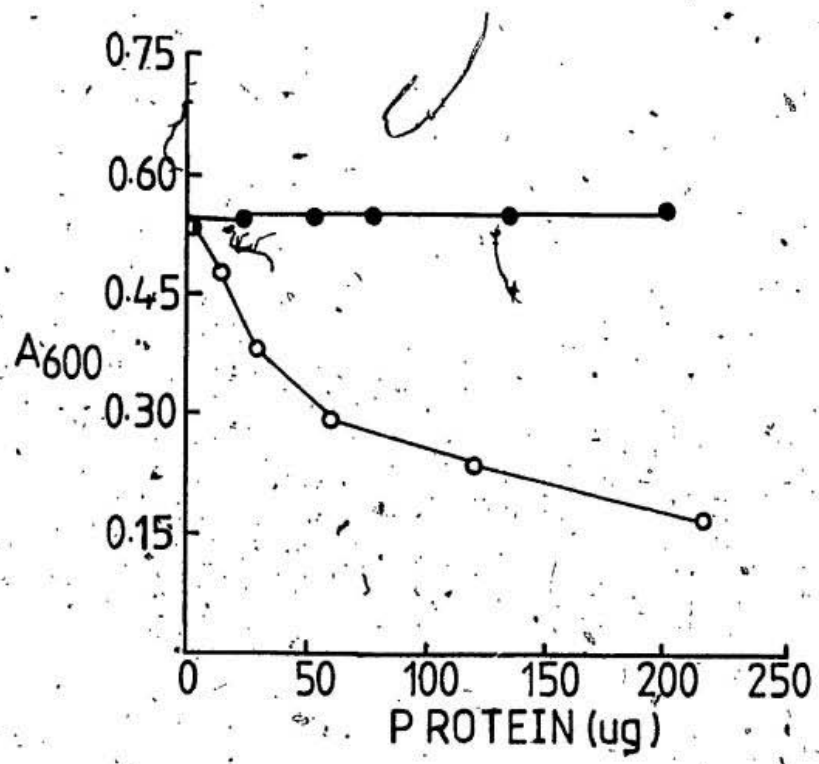


Fig. 20. Comparison of the effect of desialylated PCBP with PCBP on heparin-VLDL precipitation reaction.

Assays were carried out using VLDL (100µl; 220µg protein) either in the presence of increasing amounts of PCBP (O) or desialylated PCBP (●) and were performed using 10 mM Ca^{2+}. Other details of the assay are as described in legend to Fig. 7.

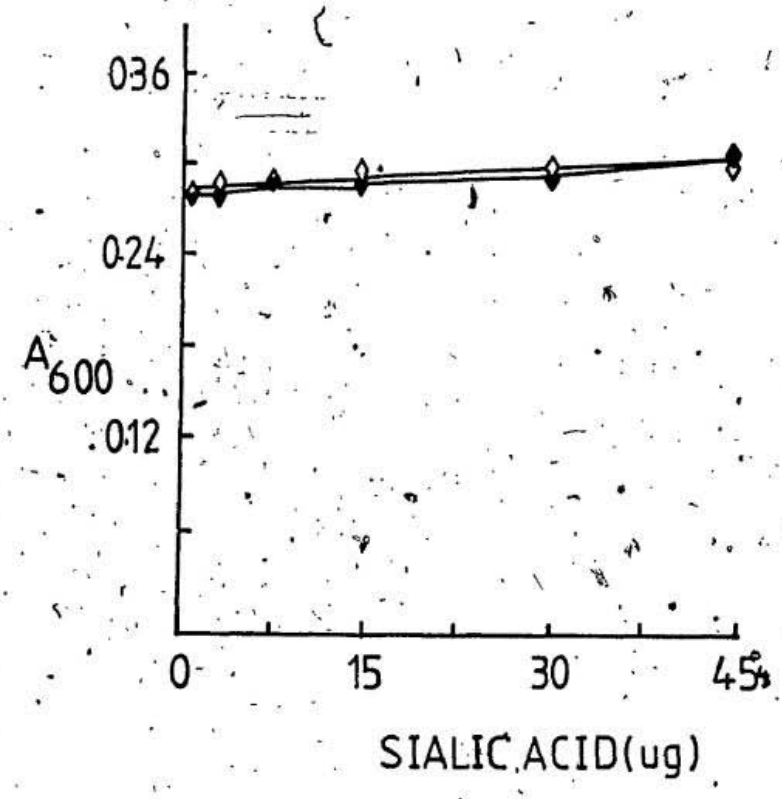


Fig. 21. Effect of addition of increasing amounts of sialic acid on heparin-VLDL precipitation reaction.

Assays with increasing amounts of sialic acid in the absence (◊) or presence (◆) of desialylated PCBP were carried out using VLDL (50 µl; 110 µg protein). Both assays were performed at 10 mM Ca^{2+}. Other details are as described in the legend to Fig. 7.

(ii) FP

Treatment of FP with neuraminidase resulted in a partial desialylation (Table 6). The desialylated FP on PAGE (139), appeared as a slower moving band distinct from FP (Fig. 22). Desialylated FP retained its ability to bind to Sepharose-phenylphosphorylcholine affinity adsorbent and could be eluted with a phosphorylcholine gradient (Fig. 23). But, the desialylated FP when added to precipitation assays with human VLDL showed reduced inhibitory effect compared to native FP (Fig. 24).

E. PAGE and immunodiffusion analysis of PCBP isolated from control and inflamed rats.

PAGE (139) profiles of PCBP from control serum and serum from inflamed rats and CRP isolated from inflamed rabbit serum are shown in Fig. 25. The mobility of PCBP isolated from inflamed rat serum is identical to PCBP isolated from control rat serum but is distinctly different from rabbit CRP. Immunochemical reactivity of serum from inflamed rats against antiserum to PCBP and antiserum to rabbit CRP was analysed in agarose gels (Fig. 26). Inflamed rat serum produced a single precipitin line in identity with control serum and purified PCBP from control rat serum. However serum from inflamed rats failed to cross-react against antiserum to rabbit CRP.

Fig. 22. PAGE of purified FP and desialylated FP PAGE was performed on 7% polyacrylamide gels according to the method of Davis (139). Gel A: purified FP (15 µg protein), Gel B: mixture of purified FP and desialylated FP (15 µg of each protein) and Gel C: desialylated FP (15 µg protein).


+
-
A
B
C

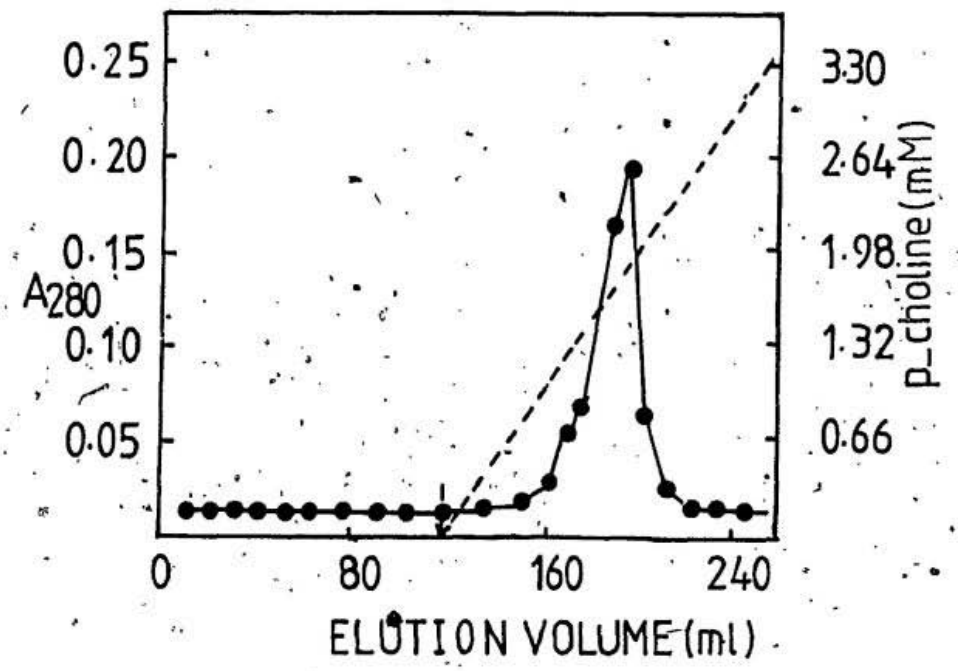


Fig. 23. Gradient elution of desialylated FP from Sepharose-phenylphosphorylcholine affinity adsorbent by P-choline.

Desialylated FP (2.90 mg protein) was applied on the affinity column and eluted by a P-choline gradient (0-3.3 mM). The desialylated FP was eluted at 1.85 mM P-choline and the recovery was 91%.

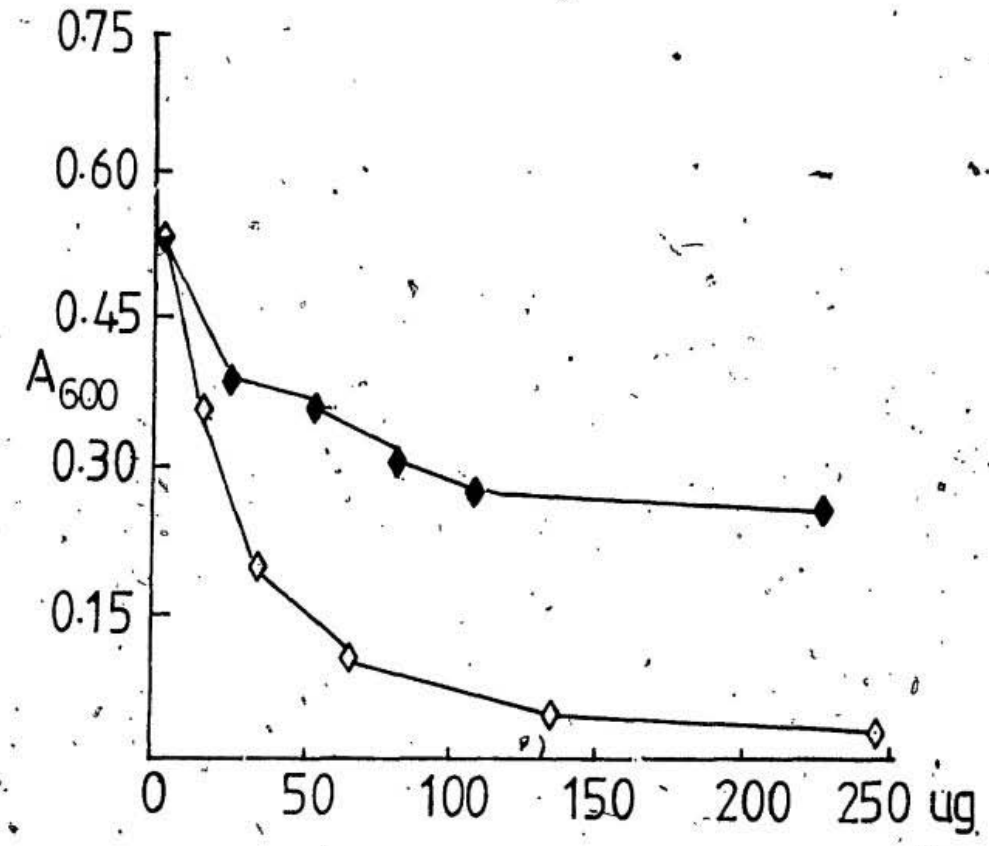


Fig. 24. Comparison of the effects of addition of increasing amounts of FP and desialylated FP on heparin-VLDL precipitation reaction.

Assays with increasing amounts of either FP (◇ - ◆) or desialylated FP (◆ -) were carried out using VLDL (100 µl; 220 µg protein). Both assays were performed at 10 mM Ca^{2+}. Other details are as described in the legend to Fig. 7.

Fig. 25. PAGE of purified PCBP and rabbit CRP. PAGE was performed on 7% polyacrylamide gels according to the method of Davis (139). Gel A: rabbit CRP (15 µg); Gel B: mixture of rat PCBP from normal serum (15 µg) and rabbit CRP (15 µg); Gel C: rat PCBP from inflamed serum (15 µg).


+
−
A
B
C

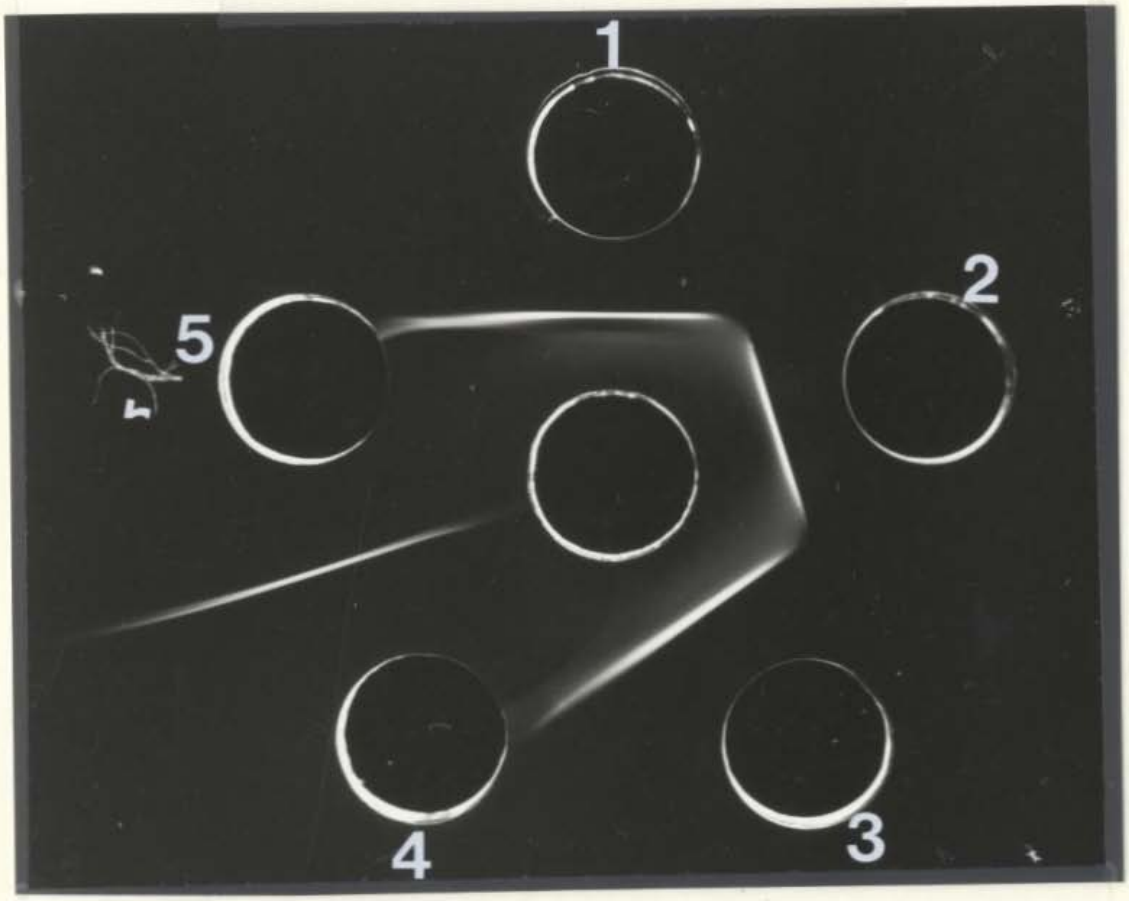


Fig. 26. Ouchterlony double-diffusion analysis of serum from control rats and inflamed rats and rabbits against antiserum to PCBP and rabbit CRP. Centre well, antiserum to PCBP; well 1 normal rat serum; well 2, purified PCBP from control rat serum; well 3, serum from inflamed rat; well 4, antiserum to rabbit CRP; well 5, serum from inflamed rabbit.

3.3. Discussion

PCBP has previously been shown to inhibit heparin-lipoprotein precipitation in the presence of Ca^{2+} (131). The results presented in the earlier study showed a stoichiometric relationship between the concentrations of VLDL and PCBP in the precipitation reaction. It was suggested that PCBP may interact with PC molecules exposed on the surface of VLDL and thereby interfere with the participation of the phospholipids in the precipitation reaction resulting in the inhibition of lipoprotein precipitation (131). Other phosphorylcholine binding pentraxins viz. FP, rabbit/human CRP were expected to demonstrate a similar inhibitory effect on lipoprotein precipitation. However the present study reveals an important difference between glycosylated pentraxins (PCBP and FP) and non-glycosylated pentraxins (rabbit and human CRP) with respect to their effects on heparin-lipoprotein precipitation reaction. FP, like PCBP inhibited the precipitation reaction.

The inhibition of precipitation reaction by PCBP was demonstrated by its addition to assays with CRP-depleted rabbit serum, normal rabbit serum and human VLDL and LDL (Fig. 11 and 14). Furthermore assays with serum from inflamed rats which had about two fold increase in PCBP levels over control serum showed decreased precipitation reactions compared to normal rat serum and required the addition of about 32μM P-choline to counter the inhibitory effect of PCBP (Fig. 7).

These results obtained from a variety of experiments indeed confirm the ability of PCBP to inhibit lipoprotein precipitation reaction.

In the case of FP, an inverse relationship between FP levels and precipitation reaction was observed. The presence of FP in female hamster serum reduced the lipoprotein precipitation reaction since its depletion from this serum led to an increase in the precipitation reaction (Fig. 9). The marked difference in lipoprotein precipitation reaction between male and female serum may be attributed largely to the presence of FP in the female serum, since the triglyceride and cholesterol levels in control male and female serum were nearly identical (Table 5). DES treated male hamster serum had increased levels of FP compared to testosterone treated female hamster serum and consequently showed decreased lipoprotein precipitation compared to testosterone treated female hamster serum (Fig. 10). Finally, the direct addition of FP to precipitation assays with male hamster serum, FP-depleted female hamster serum, human VLDL and LDL showed a clear inhibitory effect of FP (Fig. 13 and 14) on the precipitation reaction.

In contrast to PCBP and FP, rabbit and human CRP did not inhibit the precipitation reaction. Serum from inflamed rabbits contained significantly increased CRP levels in comparison to control serum, yet it showed markedly increased lipoprotein precipitation (Fig. 8) compared with assays.

using control serum. Furthermore, the addition of rabbit CRP to assays with PCBP-depleted serum, CRP-depleted rabbit serum and of human or rabbit CRP to assays with human VLDL and LDL showed no inhibitory effect (Fig. 12 and 14). In fact the addition of rabbit CRP to assays with either CRP-depleted rabbit serum, (Fig. 12 and 14) human VLDL or LDL and human CRP to VLDL and LDL showed a small increase in the precipitation reaction.

A rationale for the lack of inhibition by human or rabbit CRP became apparent when the role of sialic acid residues of PCBP and FP in lipoproteins precipitation was investigated. Desialylation of PCBP resulted in complete loss of its inhibitory effect (Fig. 20), while the inhibitory effect of desialylated FP was markedly reduced (Fig. 24). Desialylation did not cause any appreciable change in PCBP. This was indicated by three lines of evidence. First, desialylated PCBP continued to bind to Sepharose-phenylphosphorylcholine affinity adsorbent and was eluted by P-choline at a concentration identical to that required for elution of native PCBP (Fig. 17). This indicates that desialylation does not affect the phosphorylcholine binding property of PCBP. Second, the CD spectra of desialylated and native PCBP essentially the same (Fig. 19) suggesting that no detectable denaturation had occurred on desialylation within the limits of analysis performed. Third, the desialylated PCBP cross-reacted against antiserum to native PCBP and maintained a continuous line of immunochemical

identity with PCBP (Fig. 18). Similarly the partially desialylated FP maintained its ability to bind to Sepharose-phenylphosphorylcholine affinity adsorbent (Fig. 23). Therefore it may be concluded that the observed loss of inhibitory effect of desialylated PCBP and FP was not due to any denaturation of these proteins by neuraminidase treatment. The data showing the loss of inhibitory effect upon desialylation (Fig. 20 and 24) is, therefore, interpreted to suggest that the sialic acid residues on PCBP and FP play some role to express their inhibitory effect. In addition, the failure of human and rabbit CRP to inhibit the precipitation reaction clearly indicates that the inhibitory effect is not due to phosphorylcholine binding property alone.

A critical role for negatively charged sialic acid residues is consistent with the postulated ionic nature of glycosaminoglycan-lipoprotein interactions (53,63,64). Studies from other laboratories have shown an inverse relationship between the sialic acid content of apo B in LDL and the extent of interaction with glycosaminoglycans and proteoglycans (153,154,155). The negatively charged sialic acid residues on LDL have been ascribed to prevent closer interactions between sulphated heparin and LDL with consequent reduction in LDL precipitation (154). It is therefore not surprising that the sialic acid residues on PCBP and FP play a critical role in the inhibition of heparin-lipoprotein precipitation reaction.

Although sialic acid residues are critical to observe the

inhibitory effect, it appears that the mere presence of sialic acid residues on the pentraxin is not sufficient for inhibition. Evidence to support this possibility comes from the observation that purified human SAP, a sialic acid containing pentraxin that does not bind phosphorylcholine, had no effect on the VLDL heparin precipitation reaction (Fig. 15). Similarly, fetuin, a glycoprotein which does not bind phosphorylcholine had no effect on the precipitation reaction (Fig. 15). Addition of free sialic acid in the presence or absence of desialylated PCBP to assays with VLDL showed no effect on the precipitation reaction (Fig. 21). Considered together, these results indicate that phosphorylcholine binding property and the sialic acid residues are both required on the proteins in the molecular process of inhibition. PCBP and FP, but not rabbit and human CRP or human SAP, fulfill both these requirements.

A mechanism to account for both the requirements (ie. phosphorylcholine binding ability and sialic acid residues) in the process of inhibition of lipoprotein precipitation by pentraxins may be proposed. It is conceivable that phosphorylcholine binding glycosylated pentraxins may bind to the phospholipids on the surface of the lipoproteins. The binding of sialylated pentraxins to the lipoprotein may lead to electrostatic repulsion between negatively charged sialic acids and sulphate groups of heparin. This in turn may decrease the electrostatic attractions between sulfate

groups of heparin and the lipoproteins with consequent reduction in lipoprotein precipitation.

An alternate explanation consistent with the present data, is that the binding of pentraxins to lipoprotein may involve the interaction of sialic acid residues with the positively charged sites on apo B and apo E. Such an interaction may interfere with the interaction of lipoproteins with heparin. It may be recalled that positively charged arginine and lysine residues on apo B and apo E containing lipoproteins are believed to be involved in the interaction of heparin with lipoproteins (reviewed in Chapter 1). With the present data it is not possible to distinguish whether one or both the above cited mechanisms are operative.

It may be pertinent to add here that the interaction of lipoproteins with sulfated polysaccharides in the presence of divalent cations is known to produce both soluble and insoluble complexes (63). In the present studies the complex formation is monitored by a turbidimetric assay which gives an estimation of only insoluble complex formation. Therefore the effects of pentraxins on the formation of soluble heparin-lipoprotein complexes cannot be determined in these studies.

The reason for the increase in lipoprotein precipitation upon addition of rabbit CRP to assays with CRP-depleted inflamed rabbit serum and of human or rabbit CRP to assays with human VLDL and LDL is not well understood (Fig. 12 and 14). Human or rabbit CRP alone did not precipitate with

heparin and calcium. Cabana et al. (156) have reported the binding of human and rabbit CRP to heparin immobilized on agarose in the presence of Ca^{2+} (1-50 mM) at low ionic strength (0.01 M Tris, pH 7.4). Furthermore, under these conditions, VLDL and LDL were also bound to heparin-agarose. The interaction of CRP with lipoproteins in the presence of heparin and Ca^{2+} may explain the increase in precipitation reaction observed in the present study. It is conceivable that such interactions between CRP, lipoproteins and heparin may promote the formation of insoluble complexes. However, further work is required to support the possibility. It may be pertinent to add that previous studies (131) have failed to show the binding of PCBP to heparin immobilised on Sepharose.

The present study also shows that addition of human SAP to assays with VLDL produced an increase in the absorbance (Fig. 15). This increase may be attributed to the well recognized aggregation of SAP in the presence of Ca^{2+} (157). In addition SAP has also been shown to interact with heparin in a Ca^{2+}-dependent (0.5-1.0 mM) reaction (158). However, further work is necessary to support these possibilities.

The present studies showed two other differences between PCBP and rabbit CRP with regards to the normal serum concentrations of these two pentraxins and their behaviour as acute phase reactants. From virtually undetectable (or trace) levels in control serum, rabbit CRP levels increased dramatically in serum from inflamed rabbits (Table 5). In

contrast, PCBP is present in significant amounts in control serum and its concentration upon inflammation increases by only two fold (Table 5). The presence of PCBP, in inflamed rat serum was confirmed by PAGE and immunodiffusion analysis (Fig. 25 and 26).

During the course of this study changes in serum lipid levels have been observed in rabbits that accompany inflammation. On inflammation a dramatic increase in serum triglyceride and cholesterol levels is seen in rabbit serum (Table 5). This increase may largely explain the increase in lipoprotein precipitation observed when inflamed rabbit serum is used (Fig. 8a). As stated before, the interactions between CRP and lipoproteins in the presence of heparin and Ca^{2+} may also contribute to the increase in precipitation reaction. Inflammation-induced changes in plasma lipid levels in rabbits have also been observed by Cabana et al. (159). These studies show that during inflammation, in rabbit plasma, the VLDL and LDL levels increase while the HDL levels decrease. In contrast to rabbits, inflammation did not induce any increase in the lipid levels in rat serum (Table 5).

In the present study increases in serum lipid levels have also been observed in the male and female hamster serum on treatment with DES and testosterone respectively (Table 5). The influence of sex steroids on plasma lipoprotein levels is well documented (160,161).

In conclusion, the present studies have revealed a striking difference in the effects of glycosylated pentraxins (PCBP and FP) and non-glycosylated pentraxins (rabbit and human CRP) towards heparin-lipoprotein precipitation reaction. Furthermore this difference may be attributed to the presence of sialic acid residues on PCBP and FP.

Chapter 4

INTERACTION OF PCBP WITH LIPOSOMES.

4.1. Introduction

PCBP has a known binding specificity for phosphorylcholine. This property of PCBP was used in its isolation from rat serum (131). Evidence for an interaction between the phosphorylcholine groups on the surface of the lipoproteins and PCBP was presented in previous studies. In these studies, treatment of VLDL with phospholipase C reversed the inhibitory effect of PCBP on heparin-VLDL precipitation reaction. These observations emphasized the importance of phosphorylcholine groups in the process of inhibition by PCBP. However these experiments provided only indirect evidence for the interaction between phospholipids in lipoproteins and PCBP. It was, therefore, of interest to examine the ability of PCBP to bind with phospholipids having phosphorylcholine as head groups. Liposomes have often been used as model systems to study the interaction of proteins with cell membranes as well as to study apoprotein-phospholipid interactions (162,163). The studies presented in this chapter were undertaken to characterize the binding of PCBP to liposomes composed of egg yolk phosphatidylcholine and provide insight into the nature and specificity of PCBP-phospholipid interaction.

Results

4.2

A. Binding of radioiodinated PCBP to Sepharose-phenylphosphorylcholine affinity adsorbent and PAGE analysis of purified ^{125}I-PCBP.

Radioiodinated PCBP was applied to a Sepharose-phenylphosphorylcholine column (5 ml bed volume). The bound ^{125}I-PCBP (57% of the counts applied) was eluted with 4 mM P-choline, and appeared as a single peak from the column (results not shown). This indicated that radioiodination of PCBP had not affected its binding property for phosphorylcholine ligand. The purity of radioiodinated PCBP was established by electrophoresing ^{125}I-PCBP (0.9 µg) diluted with 16 µg of PCBP on polyacrylamide gel according to the method of Davis (139). The gel after staining and destaining was sliced into 5 mm sections and each section assayed for radioactivity. Over 80% of the counts applied appeared as a single peak corresponding to unlabelled PCBP (Fig. 27). On the basis of these observations it may be assumed that radioiodinated PCBP is similar to PCBP.

B. Requirement of BSA to prevent non-specific binding of ^{125}I-PCBP to the assay tube in the presence of Ca^{2+}.

Preliminary experiments to establish the assay conditions for the binding of ^{125}I-PCBP to liposomes showed considerable binding of ^{125}I-PCBP to the walls of the assay tube in the

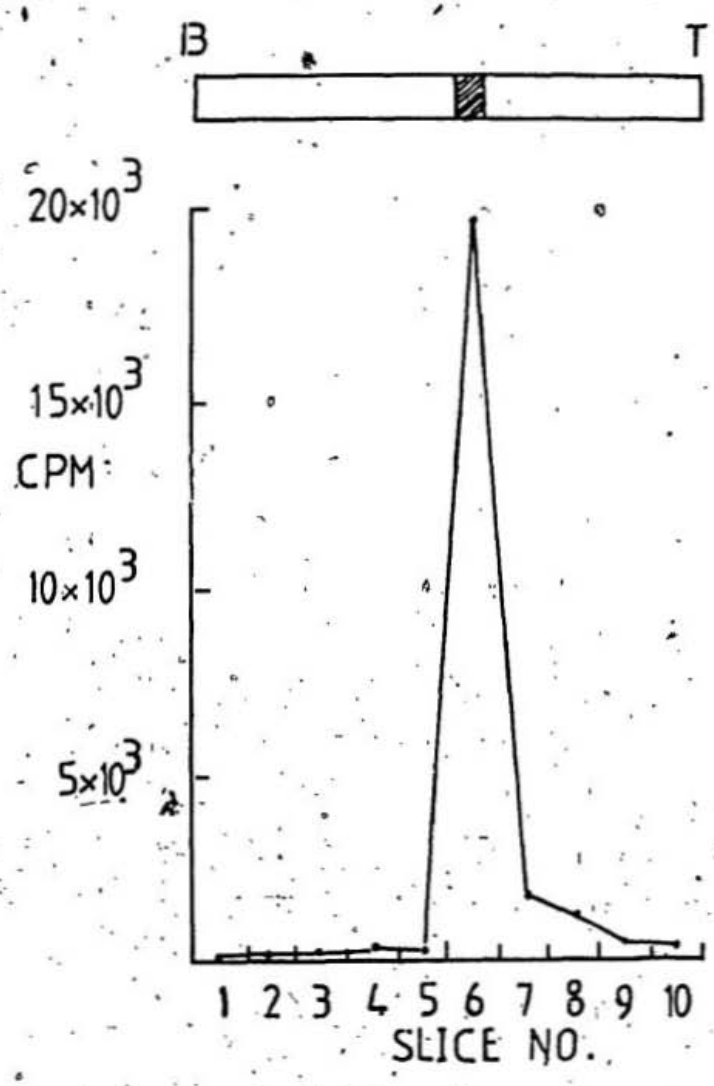


Fig. 27. Criteria of purity of ^{125}I-PCBP by polyacrylamide gel electrophoresis. ^{125}I-PCBP (0.9 µg) was diluted with PCBP (16 µ g) and analyzed on 7% polyacrylamide gels according to the method of Davis (139). The gel was stained with Coomassie blue for 12h, destained electrophoretically, and then sliced into 5 mm sections and counted for radioactivity. B, bottom; T, top of gel.

presence of Ca^{2+} (Fig. 28). This binding occurred in control assays in absence of liposomes. This non specific binding of ^{125}I-PCBP to the assay tubes was abolished by the inclusion of 0.1% BSA (w/v) in the assay mixture (Fig. 28). The use of either fatty acid free BSA or BSA did not affect the binding of PCBP to liposomes, hence 0.01% BSA was routinely used in all assays.

c. Effect of Ca^{2+} on the binding of PCBP to liposomes composed of 75% PC and 25% LPC.

Fig. 29a shows the binding of ^{125}I-PCBP to liposomes as a function of Ca^{2+} concentration. In the absence of Ca^{2+} about 20% of PCBP added was bound to liposomes and the binding increased with increasing amounts of Ca^{2+}. A study on the binding of PCBP to liposomes as a function of PCBP concentration either in the absence or presence of 10 mM Ca^{2+} showed that the binding to the liposomes was three to five fold greater in presence of Ca^{2+} compared to the binding in the absence of Ca^{2+}. In the presence of Ca^{2+}, the binding increased progressively and appeared to saturate in the range of 300-400 µg PCBP. However, in the absence of Ca^{2+}, the binding increased up to addition of 100 µg PCBP, but thereafter remained unchanged up to 400 µg PCBP (Fig. 29b).

The affinity of the binding sites on liposomes for PCBP in the presence of Ca^{2+} was estimated using Scatchard analysis (164) of the binding data. The Scatchard plot was linear

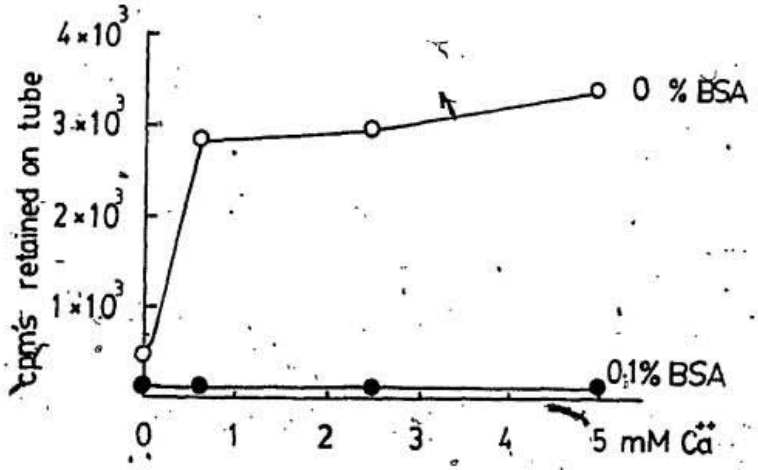


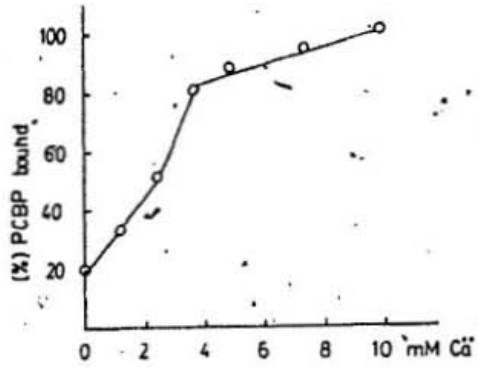
Fig. 28. Requirement of BSA to prevent non-specific binding of ^{125}I-PCBP to the walls of the assay tube. Assay mixture (0.5 ml) contained 15 mM Tris-HCl, (pH 7.8), 0.9 µg ^{125}I-PCBP, increasing amounts of Ca^{2+} either in the presence (●) or absence (O) of 0.1% w/v BSA. After incubation with shaking at 37°C for 1h, the tubes were centrifuged, supernatant removed and the tubes were counted for ^{125}I-activity.

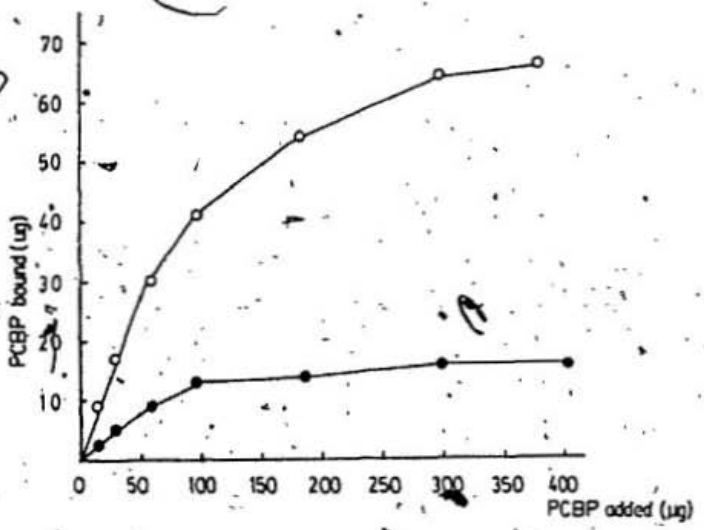
Fig. 29. Effect of Ca^{2+} on the binding of ^{125}-PCBP to liposomes.

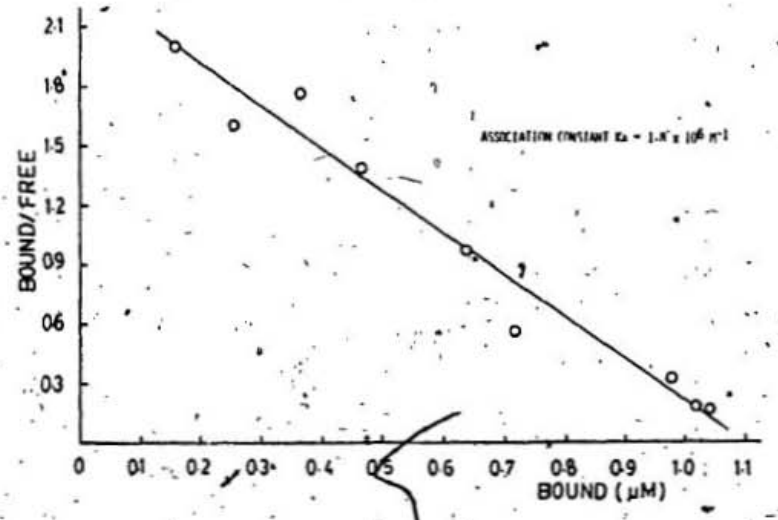
a) Assay mixture contained liposomes composed of 75% PC, 25% LPC (600 nmol of phospholipid), 0.9 µg of ^{125}I-PCBP, 96 µg PCBP, 0.1% (w/v) BSA and indicated amounts of Ca^{2+}. Other conditions for the assay were as described in legend to Fig. 28. The amount of PCBP bound was calculated from ^{125}I-activity associated with the pellet after centrifugation. The recovery of the phospholipid in pellets was quantitative. The maximum amount of PCBP bound (42 µg) has been assigned as 100% and the PCBP bound at different Ca^{2+} concentrations is expressed as the percentage of maximum bound.

b) Assays were carried out either in the presence ( ) or absence ( ) of 10 mM Ca^{2+} and contained liposomes composed of 75% PC and 25% LPC (600 nmol phospholipid), 0.1% (w/v) BSA, ^{125}I-PCBP (0.9 µg) with increasing amounts of PCBP. Other details of the assay were as described in legend to Fig. 29a. The amount of PCBP bound was calculated as described in legend to Fig. 29a.

c) shows Scatchard analysis of binding of PCBP to liposomes. The amounts of bound and free PCBP were estimated from a saturation curve (data not shown) and the data plotted. The straight line was drawn by the method of least squares linear regression.


(%) PCBP bound
100
80
60
40
20
0
2
4
6
8
10 mM Ca


PCBP bound (µg)
70
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
PCBP added (µg)


BOUND/FREE
2·1
1·8
1·5
1·2
0·9
0·6
0·3
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
BOUND (µM)

and the association constant (Ka) calculated from the slope of the Scatchard plot gave a value of $1.8 \times 10^6 M^{-1}$ (Fig. 29c). The total number of binding sites on the liposomes were calculated from the intersection of the line with the abscissa and gave a value of 0.96 nmol/ µmol of phospholipid. This value compares favourably with the total binding capacity of 0.86 nmol/ µmol of phospholipid obtained from the saturation binding curve (Fig. 29b).

D. Effect of LPC on the binding of PCBP to liposomes.

The binding of PCBP to liposomes containing various ratios of PC and LPC was studied as a function of PCBP concentration (Fig. 30a). The binding of PCBP to liposomes composed of PC alone was poor. The maximum binding of PCBP to the liposomes was observed when the liposomes contained 25% LPC (Fig. 30a). The recovery of phospholipid from the pellets was quantitative in liposomes composed of 100% PC and 75% PC, 25% LPC (Fig. 30b). However further increase in LPC (ie. in liposomes composed of 50% PC, 50% LPC and 100% LPC) reduced the phospholipid recovery in the liposome pellets (Fig. 30b). The amount of PCBP bound to each of liposomes when 250 µg of PCBP was added is shown in Fig. 30b.

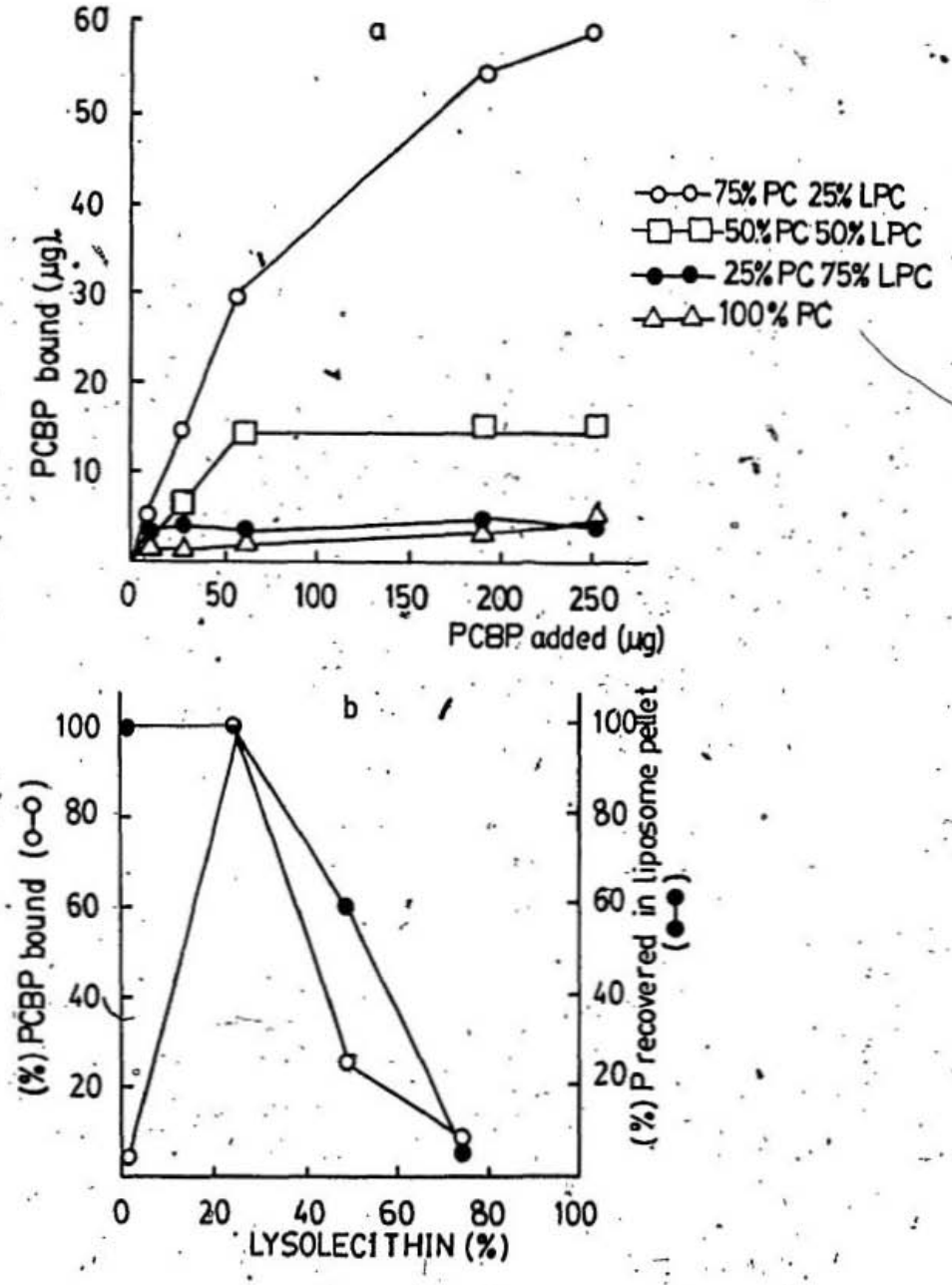
E. Binding of PCBP to liposomes as a function of liposome concentration.

Increasing amounts of PCBP bound to liposomes when

Fig. 30. Effect of LPC on the binding of PCBP to liposomes (a) and recovery of phospholipid and PCBP from the liposome pellet as a function of LPC concentration (b).

a) Binding assays were carried out in presence of 10 mM Ca^{2+}, 0.1% (w/v) BSA, 0.9 µg of ^{125}I-PCBP, increasing amounts of PCBP and liposomes (600 nmol phospholipid) of the following composition: 75% PC, 25% LPC (O); 50% PC, 50% LPC (-□-); 25% PC, 75% LPC (●) and 100% PC (Δ). The amount of PCBP bound was calculated as described in legend to Fig. 29a.

b) The liposome pellets obtained from experiment described in fig. 30a, were analyzed for phospholipid Pi following the method of Fiske and Subba Row (150). The Pi recovered has been expressed as the percentage of P recovered in the pellet. Maximum amount of PCBP bound (60µg) has been assigned as 100% and the PCBP bound to liposomes of different LPC compositions has been expressed as the percentage of maximum bound.


a
PCBP bound (µg)
PCBP added (µg)
75% PC 25% LPC
50% PC 50% LPC
25% PC 75% LPC
100% PC
b
(%) PCBP bound (o-o)
(%) P recovered in liposome pellet (•-•)
LYSOLECITHIN (%)

studied as a function of liposome concentration and the binding increased up to 1000 nmol of phospholipid after which it remained unchanged at higher concentrations of liposomes (Fig. 31). Maximum binding (about 70µg) was obtained with liposomes containing 1000-1200 nmol of phospholipid. In routine assays liposomes containing 600 nmol of phospholipid were used as this value was on the linear part of the concentration curve (Fig. 31).

F. Effect of incubation time and temperature on the binding of PCBP to liposomes.

The binding of PCBP to liposomes remained unchanged between 20 and 120 min of incubation at 37°C (Fig. 32). Therefore, an incubation time of 60 min was used in the binding assays.

The effect of incubation temperature was studied on the binding of PCBP to liposomes. The binding was slightly decreased upon incubation of the assay mixture at 4°C compared to incubation at 37°C.

G. Effect of different molecular species of phospholipids and polar head group substitutions on the binding of PCBP to liposomes.

The binding of PCBP to liposomes was unaffected when liposomes composed of different phospholipid acyl groups were used in the assay. Fig. 33a shows that the amount of

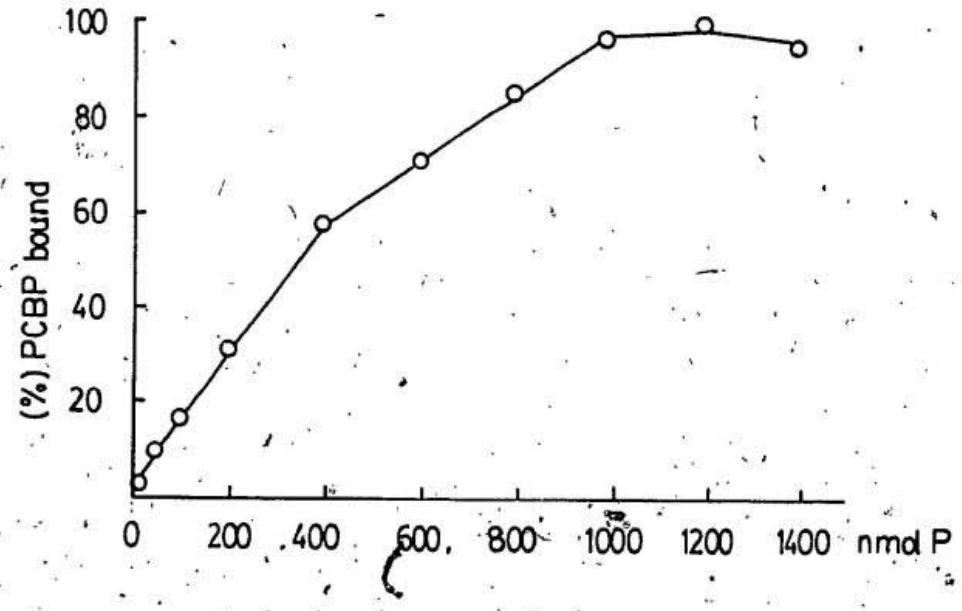


Fig. 31. Effect of increasing amounts of liposomes on the binding of PCBP to liposomes. Assay mixture contained 0.9 g of ^{125}I-PCBP, 10 mM Ca^{2+}, 0.1% (w/v) BSA, 108 g PCBP and increasing amounts of liposomes composed of 75% PC, 25 LPC. Other details of the assay as described in legend to Fig. 29a. The recovery of phospholipid in the liposome pellets was quantitative. The maximum amount of PCBP bound (70 µg) has been assigned as 100% and PCBP bound at different liposome concentrations is expressed as the percentage of maximum bound.

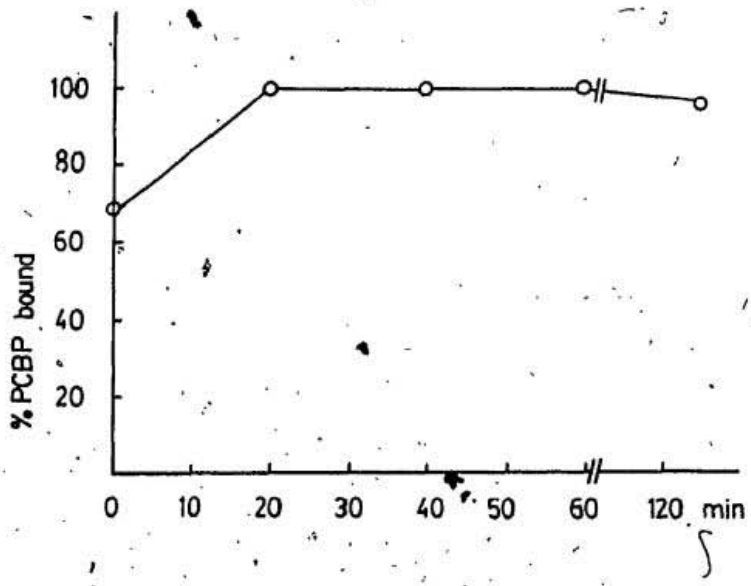


Fig. 32. Binding of PCBP to liposomes as a function of time.

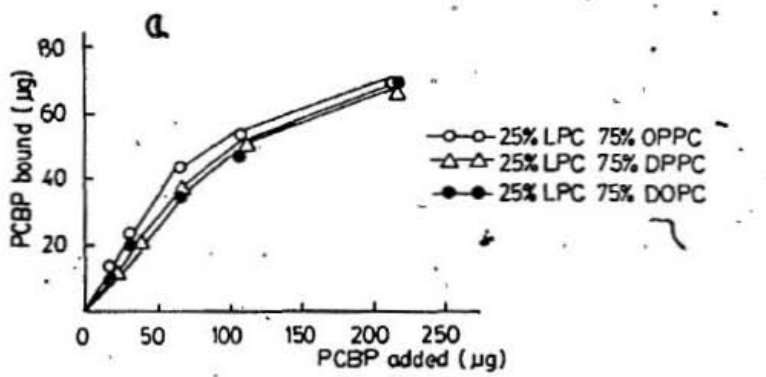
Assays consisting of 0.9 µg of ^{125}I-PCBP, 10 mM Ca^{++}, 0.1% (w/v) BSA, 126 µg PCBP and liposomes composed of 75% PC, 25% LPC (600 nmol phospholipid) were incubated for various times at 37°C. The tubes were centrifuged for 30 min and pellets were counted for ^{125}-I activity. The maximum amount of PCBP bound (44 µg) has been assigned as 100% and PCBP bound at various times has been expressed as percentage of maximum bound.

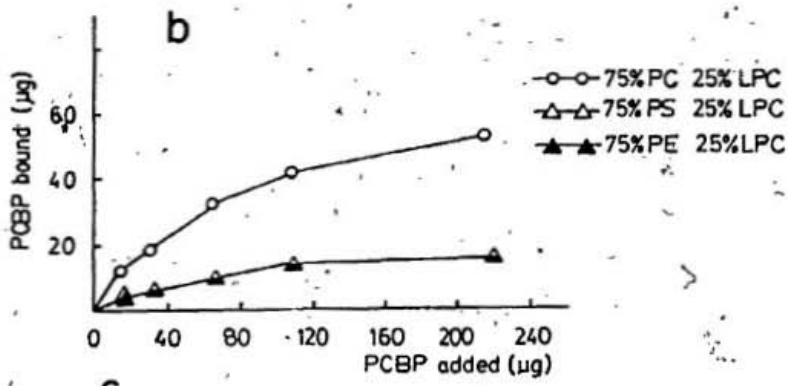
Fig. 33. Effect on the binding of ^{125}I-PCBP to liposomes composed of different acyl groups of the phospholipids (a) and of phospholipids having different polar head group (b).

a) Assays consisted of 0.9 µg ^{125}I-PCBP, 10 mM Ca^{2+}, 0.1% (w/v) BSA, increasing amounts of PCBP and liposomes (600 nmol phospholipid) containing 25% LPC, 75% 1-oleoyl-2-palmitoylphosphatidylcholine (O) 25% LPC, 75% Dipalmitoyl-phosphatidylcholine (Δ) and 25% LPC, 75% dioleoylphosphatidylcholine (●). Other conditions of the assay are as described in legend to Fig. 28.

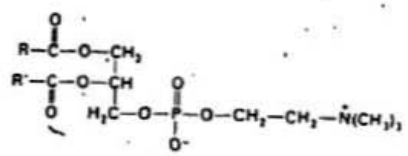
b) Assays were carried out in presence of 0.9 µg ^{125}I-PCBP, 10 mM Ca^{2+}, 0.1% (w/v) BSA, increasing amounts of PCBP and liposomes containing 75% PC, 25% LPC (O), 75% phosphatidylserine, 25% LPC (Δ) and 75% phosphatidyl-ethanolamine, 25% LPC (▲). Amount of PCBP bound to the liposomes was calculated as described in legend to Fig. 29a.

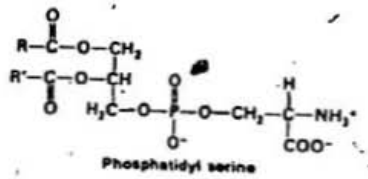
c) Structures of phospholipids used to study the binding of PCBP to liposomes. Liposomes composed of phospholipids having different polar head groups were used to study the binding of PCBP to liposomes as described in legend to fig. 33b.

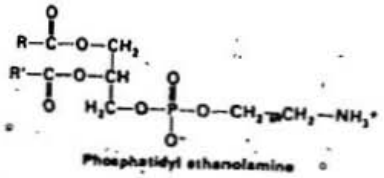




c


Phosphatidyl choline


Phosphatidyl serine


Phosphatidyl ethanolamine

PCBP bound to liposomes composed of three different molecular species of phospholipids viz. dioleoyl-, dipalmitoyl-, and 1-oleoyl-2-palmitoyl- was similar. In contrast, the binding of PCBP to liposomes was significantly affected when polar head groups other than PC were used (Fig. 33b). Substitution of phosphorylcholine head groups of PC by phosphorylethanolamine and phosphorylserine in the liposomes reduced the binding substantially.

H. Inhibition of binding of PCBP to liposomes by P-choline.

The binding of PCBP to liposomes was studied in the presence of P-choline. The binding was progressively inhibited by increasing concentrations (0-400μM) of P-choline (Fig. 34).

4.3. Discussion

The data obtained in this study suggests that PCBP can bind to multilamellar liposomes composed of PC and LPC. Despite the binding specificity of PCBP for phosphorylcholine, it bound poorly to liposomes containing phosphatidylcholine alone (Fig. 31a). A definite amount of LPC (25%) was required to be incorporated in the liposomes for maximal binding of PCBP. This requirement of LPC may be attributed to constraints on the orientation and accessibility of the polar head groups of PC imposed by intermolecular interactions in PC containing liposomes (165). Inclusion of LPC at low concentrations may reduce the intermolecular interactions

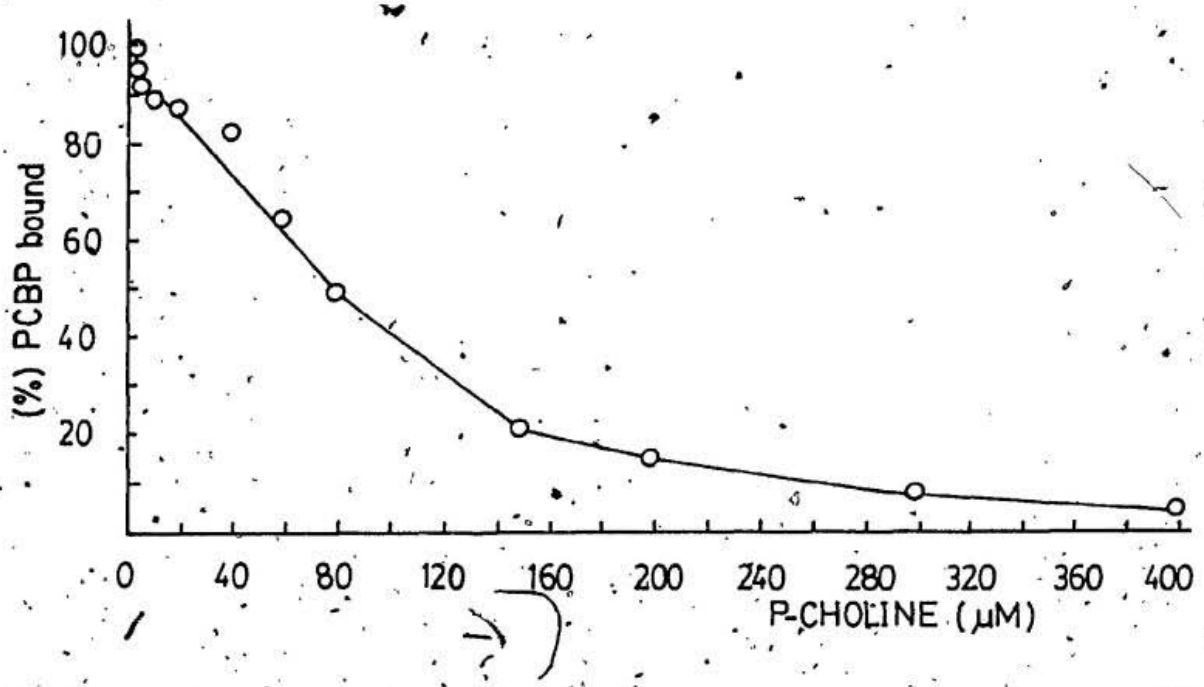


Fig. 34. Effect of phosphorylcholine on the binding of ^{125}I-PCBP to liposomes. The incubation mixture contained 0.9 μg ^{125}I-PCBP, 10 mM Ca^{2+}, 0.1% (w/v) BSA, liposomes composed of 75% PC, 25% LPC (600 nmol of phospholipid), 126 μg PCBP and increasing amounts of P-choline. The maximum amount of PCBP bound (48 μg) in the absence of P-choline has been assigned as 100% and the PCBP bound at different P-choline concentration has been expressed as percentage of maximum bound.

such that the polar head groups become more accessible for binding of PCBP. However, the possibility that the binding of PCBP is mediated directly through the polar head groups of LPC cannot be excluded. Attempts to examine this possibility were unsuccessful due to the poor recovery of phospholipid from the liposome pellet at higher concentrations of LPC (Fig. 30b). Consequently the PCBP bound either to PC or LPC would be lost in the supernatant after sedimentation. It is likely that LPC caused solubilization of the liposome pellet and thus reduced the phospholipid recoveries in the pellet.

Direct evidence for the importance of phosphorylcholine head groups in the binding of PCBP to liposomes comes from the studies with liposomes composed of different head groups and LPC (Fig. 33b). Liposomes containing phosphorylcholine as the head group bound three to four times more PCBP than those containing phosphorylserine or phosphorylethanolamine head groups. Replacement of choline with ethanolamine or serine greatly reduces the binding (Fig. 33b). The enhanced binding of PCBP to phosphorylcholine polar head group containing liposomes compared to those containing phosphorylethanolamine or phosphorylserine suggests a specific involvement of phosphorylcholine head groups in the binding of PCBP. It is of course possible that phosphorylcholine head group may be more accessible for binding of PCBP than phosphorylethanolamine or phosphorylserine and result in enhanced binding.

The binding site for phoshorylcholine in human and rabbit CRP has been shown to consist of a primary locus for binding the phosphorylester group of phosphorylcholine and a secondary locus for binding the quaternary ammonium group (91). The binding of the phosphoryl group of phosphorylcholine to CRP requires the presence of Ca^{2+} (88). If PCBP binds to phosphorylcholine head group by a similar two loci mechanism as proposed for CRP, it may explain the enhanced binding of PCBP to liposomes in the presence of Ca^{2+} (Fig. 29a and b). Alternatively Ca^{2+} may act allosterically and not participate in the binding site of PCBP. With the present data it cannot be established whether calcium is acting allosterically or is a component of the binding site of PCBP.

In contrast to the importance of the polar head group, variation of fatty acyl chains on phospholipids did not affect the binding of PCBP to liposome (Fig. 33a). Similarly, binding of PCBP was not significantly affected by variation of incubation temperature (37° and 4°C).

One of the requirements proposed for PCBP to express the inhibitory effect on heparin-lipoprotein precipitation was its ability to bind to phospholipids on the lipoprotein (Discussion, Chapter 1). The specific binding of PCBP to PC:LPC containing liposomes with relatively high affinity ($Ka = 1.8 \times 10^6 M^{-1}$, Fig. 29c) together with the other data presented here suggest the possibility of an interaction between PCBP and lipoproteins.

Although phosphatidylcholine is an important constituent of all plasma lipoproteins, the present data suggest that surface orientation of the polar phosphorylcholine head groups may be a determining factor for the interaction of lipoproteins with PCBP. A difference in the surface orientation or accessibility of phosphorylcholine head group in various lipoproteins may arise due to differences in the phospholipid and apoprotein composition of the lipoproteins. It may be relevant to add here that in LDL about 20% of the phosphorylcholine groups are immobilized by interaction with apo B, whereas in HDL there appear to be no immobilizing interactions between phospholipid head groups and apoproteins (166,167,168).

Human CRP has been shown to bind to liposomes of various compositions (107,108,109,169). Mold et al (109) have reported the binding of CRP to positively charged liposomes composed of stearylamine, dimyristoylphosphatidylcholine, galactosylceramide and cholesterol. The binding of CRP to such liposomes was Ca^{2+} independent, occurring in the presence of EDTA and in fact inhibited by high concentrations of Ca^{2+}. In addition binding was not inhibited by phosphorylcholine. Dimyristoyl phosphatidylethanolamine could be substituted for dimyristoyl phosphatidylcholine without affecting the binding of CRP to liposomes. On the basis of these data the authors concluded that binding of CRP to positively charged liposomes was through the putative

polycation binding site of the protein (Reviewed in Chapter 1) the polycationic ligand being provided by stearylamine. Similarly, Tsujimoto et al (169) have demonstrated the binding of CRP to liposomes composed of stearylamine, dipalmitoyl- or dimyristoyl phosphatidylcholine and cholesterol.

On the other hand, Volanakis and Wirtz (170) have shown the binding of CRP to unilamellar liposomes composed of PC and LPC. In these studies, CRP failed to bind to liposomes containing PC alone and incorporation of LPC in the liposomes was necessary to demonstrate binding. The binding of CRP was Ca^{2+} dependent and could be inhibited by phosphorylcholine. In this respect, the binding of PCBP to liposomes appears to be similar to CRP.

In conclusion from the present studies it appears that the binding of PCBP to liposomes composed of PC:LPC may be mediated through a Ca^{2+}-dependent phosphorylcholine binding site on PCBP. In addition, binding through this site could be inhibited by phosphorylcholine since the results have shown that 400 μM P-choline virtually inhibited the binding of PCBP to the liposomes (Fig. 34).

Chapter 5

STUDIES ON THE BINDING OF HUMAN PLASMA LIPOPROTEINS TO IMMOBILISED PCBP.

5.1. Introduction

The results obtained from the studies on lipoprotein precipitation reaction and its inhibition by PCBP indicated that the inhibition may be mediated by the phosphorylcholine binding property and the presence of sialic acid residues of PCBP. Furthermore the data were interpreted to suggest that binding of PCBP to the phospholipids on the surface of lipoproteins is required for the inhibition process (Chapter 3). A pivotal question raised by these studies is whether PCBP can indeed interact with plasma lipoproteins. The present study was designed to characterize the interaction of PCBP immobilized on Sepharose with human plasma lipoproteins. The molecular mechanisms involved in these interactions have been explored. These studies may shed light on the basic features involved in PCBP-lipoprotein interactions.

5.2 Results

A. Binding of VLDL to Sepharose-PCBP

Application of purified human VLDL to Sepharose-PCBP column in presence of 10 mM Ca^{2+} showed that about 38% of the VLDL (in terms of protein applied) did not bind to the column (Table 7). The rest of the VLDL (about 48% of the VLDL protein) was retained on the column and was eluted with

Table 7

Distribution of lipid and apoprotein components of VLDL in unbound and bound fractions obtained from a Sepharose-PCBP column

	Applied (mg)	Distribution	
		% in unbound	% in bound
Total protein	(0.7-2.6)	38±4.1	*48±5.6
Apo B	(0.16-1.10)	46±5.6	*69±10.20
Apo E	(0.06-0.33)	50±3.4	*57±4.20
Cholesterol	(0.15-1.08)	68±7.1	58±8.60
Cholesterol esters	(0.21-2.0)	50±8.2	60±11.9
Phospholipids	(0.64-3.75)	55±6.10	64±10.10
Triglycerides	(2.13-7.55)	66±6.80	**36±5.20

The range of VLDL applied to the column are shown in parenthesis.
Results show average values (n=5) with ± S.E. of means.
* P <0.05 compared to the unbound fraction.
**P < 0.005 compared to the unbound fraction. Statistical significance was calculated using Student's unpaired t-test. Apoprotein and lipid components were estimated by electroimmunoassay and gas liquid chromatograpy techniques respectively as described in Chapter 2.

a P-choline gradient (eluted at 68 mM P-choline). A typical elution profile of VLDL from the Sepharose-PCBP column is shown in Fig. 35. The profile showing absorbance at 280 nm, is not representative of protein mass for lipoproteins because of light scattering due to turbidity of VLDL. Table 7 shows the actual protein mass distribution in the unbound and bound fractions. A possibility existed that some VLDL may not bind to Sepharose-PCBP due to its exclusion from the column. However, the elution volume of the unbound VLDL was greater than the exclusion volume of Sepharose-PCBP column, indicating that the unbound VLDL was not excluded from the Sepharose-PCBP column. The possibility that the unbound fraction resulted due to saturation of the column was investigated by applying different amounts of VLDL to the column (Table 7). When 2.5 mg of VLDL protein was applied, 1.5 mg protein was retained on the column. Assuming this to be the capacity of the column, a sub-saturating amount (0.7 mg protein) of VLDL was applied which again resulted in an unbound (0.27 mg) and a bound (0.35 mg) fraction. These results suggest that the unbound fraction is not due to saturation of the column.

The distribution of the lipid and apoprotein components of bound and unbound fraction are also presented in Table 7. Electroimmunoassay of the apoproteins and lipid quantitation in these two fractions showed significantly higher amounts of apo B and apo E (69 and 57% respectively) but less triglyceride being recovered from the bound fraction

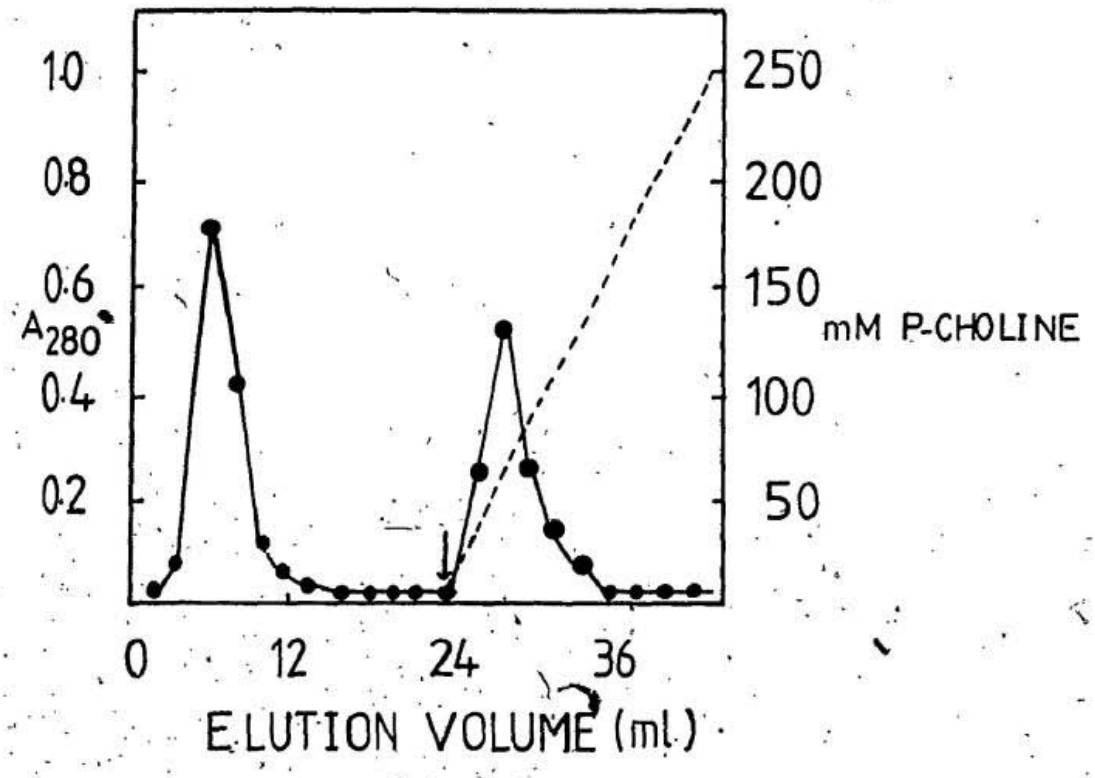


Fig. 35. Elution profile of VLDL from Sepharose-PCBP column. After the application of VLDL, the column was washed exhaustively with 0.01M Tris-HCl buffer (pH 7.4) containing 0.15M NaCl and 10 mM Ca^{2+}. The bound fraction was eluted at 68 mM P-choline on application of a P-choline gradient (0 to 250 mM). The column was operated at a flow rate of 20 ml/hr and 2 ml fraction were collected. Both the unbound and bound fractions were processed for apoprotein and lipid analysis as described in Chapter 2.

compared to the unbound fraction (Table 7). The composition of the bound and unbound fraction is presented in Table 8. The bound fraction contained more protein but less triglyceride compared to the VLDL applied (Table 8). The calculated diameters showed that the bound fraction contained VLDL of a smaller size than the unbound fraction. The decreased triglyceride content and the smaller size of the bound VLDL indicate that the bound fraction may represent a partially catabolised VLDL. Since it has been proposed that apo C levels in VLDL decrease progressively during catabolism (19), the bound and the unbound fractions were analysed for apo C II content by electroimmunoassay. Results showed that the bound fraction contained less (37% of applied) apo C II compared to the unbound fraction (50%). These results do support the possibility that the bound fraction may represent partially catabolised VLDL.

There was no binding of the VLDL to the column in absence of Ca^{2+} (Table 9). A control experiment with a blank Sepharose column showed no retention of VLDL. Elution with P-choline gradient did not elute any detectable protein from both the columns.

B. Binding of LDL to Sepharose-PCBP.

Three different amounts of purified LDL (660 µg, 750 µg and 960 µg protein) were applied to the column in the presence

Table 8

Composition of the VLDL fractions (% by weight) obtained from Sepharose-PCBP column.

	Applied	unbound+	bound
Protein	7.25±0.71	7.10±0.63	10.72±0.32*
Cholesterol	7.00±0.71	6.7±0.68	7.0±0.51
Cholesterol esters	13.0±1.3	12.2±2.1	14.3±3.1
Phospholipid	20.0±4.8	21.6±2.8	22.0±2.6*
Triglycerides	58.0±7.8	56.3±4.3	52.0±2.3*
Diameter (A°)	365±43	354±58	306±55

The percent lipid of the fractions was calculated from total lipid profiles. Results are the means ± S.E. values (n = 6).

*P < 0.05 compared to VLDL applied.

+P values between the components of the unbound and applied were not significant. Statistical significance was calculated using student's unpaired t-test.

The diameter of each fraction was estimated from the lipid profiles on the basis of the ratio of core to surface components (14).

Table 9

Lack of binding of lipoproteins to Sepharose-PCBP in the absence of Ca^{2+}.

Lipoprotein applied	% unbound
VLDL (1.4 mg protein)	94
LDL (0.8 mg protein)	92
HDL (3 mg protein)	98

Protein determination was performed by the method of Kashyap et al. (141).

of Ca^{2+} and in all cases resulted in quantaditive binding of the lipoprotein (Table 10). The LDL bound was typically eluted at 68 mM P-choline when a P-choline gradient was applied (Fig: 36). The recoveries of apo B (94%) and lipid components in the bound fraction were quantitative. This binding of LDL to Sepharose-PCBP required Ca^{2+}, since in absence of Ca^{2+} no binding was observed (Table 9).

C. Binding of HDL to Sepharose-PCBP

Binding studies of HDL to Sepharose-PCBP showed that in the presence of Ca^{2+}, about 80% HDL applied (in terms of protein) was recovered in the unbound fraction (Table 11). About 11% was bound to the column and was eluted by a P-choline gradient (at 68 mM P-choline, Fig. 37). Further elution with 1.4 M NaCl did not elute any additional lipoprotein. The apoprotein characterization of the bound and unbound fractions included the analysis for Lp(a) since Lp(a) lipoprotein is known to occur in the HDL density range (171). The results showed two important features. The bound fraction contained 88% of apo E and 97% of Lp(a) from the HDL applied. In contrast only 7.2% of Apo A-1 was recovered in the bound fraction. The analysis of protein and lipid composition of the bound and unbound fractions of HDL showed that the bound fraction contained less protein but more cholesterol, cholesterol esters and phospholipids compared to the unbound fraction (Table 12). The increase in cholesterol esters could be

Table 10

Binding of LDL to Sepharose-PCBP and percent recovery of lipids and protein in the bound fraction

	Applied (mg)	% bound
Total protein	(0.66-0.96)	94.1±4.2
Apo B	(0.61+0.89)	88.6±1.7
Cholesterol	(0.23-0.34)	91.0±8.6
Cholesterol esters	(0.89-1.3)	100.3±8.6
Phospholipids	(0.59-0.86)	86.9±5.6
Triglycerides	(0.12-0.17)	

The range of LDL applied to the column are shown in the parentheses.

Results presented are average values (n=3) with ± S.E. of means. Lipids were determined from total lipid profiles and the apo B was quantitated by electroimmunoassay.

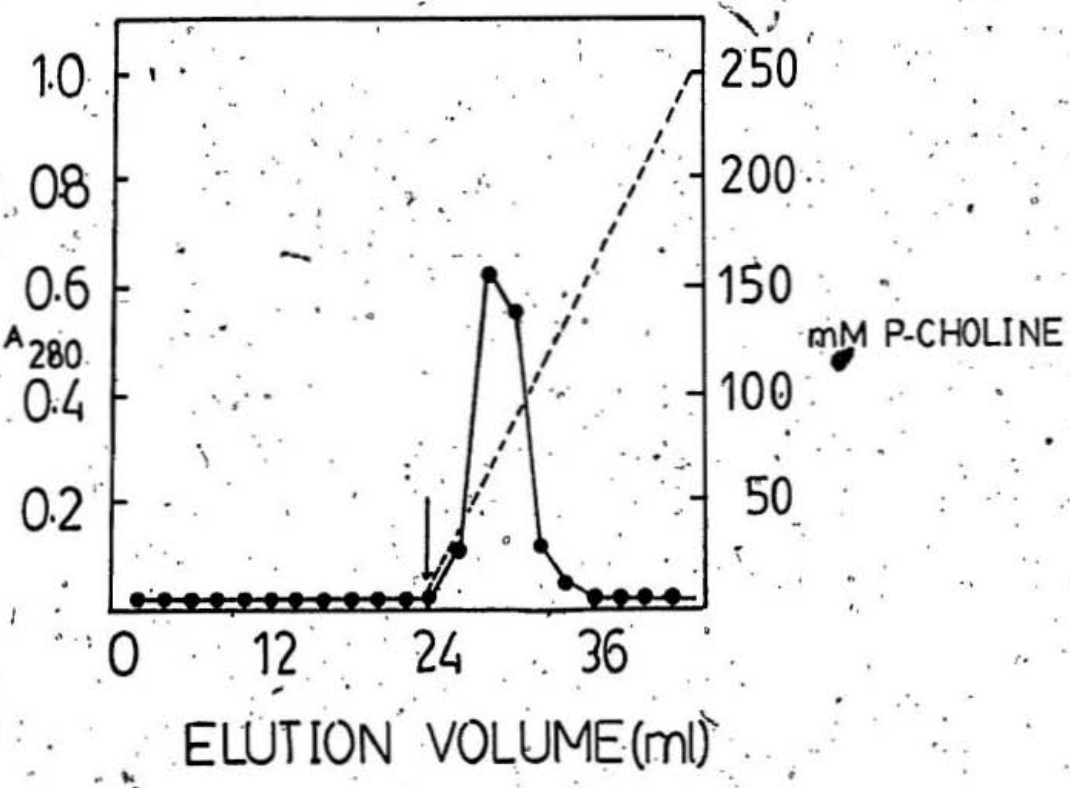


Fig. 36. Elution profile of LDL from Sepharose-PCBP column. The column and elution conditions were the same as described in Fig. 35. The bound LDL was eluted at 68 mM P-choline.

Table 11

Distribution of apoprotein and lipid components of HDL in unbound and bound fractions obtained from Sepharose-PCBP column.

	applied (mg)	Distribution	
		%unbound	% bound
Total protein	(1.5-6.0)	80±3.46	*10.9±0.57
Lp(a)	(0.02-0.08)	ND	97.5±2.48
Apo E	(0.005-0.013)	ND	88.8±6.17
Apo A-I	(0.62-2.23)	85.8±4.9	*7.26±1.15
Cholesterol	(0.056-0.116)	57±2.4	*17.6±0.75
Esterified cholesterol	(0.190-0.760)	65±2.6	*9.59±0.54
Phospholipids	(0.260-1.006)	63±2.1	*16.4±0.69
Triglyceride	(0.025-0.10)	65±2.9	*15.58±0.68

The range of HDL applied to the column is shown in the parenthesis.

Results shown are average values (n=3) with ± S.E. of means. Lipid and apo protein quantitation were performed by gas liquid chromatography and electroimmunoassay respectively. ND = Not detectable.

*, $P<0.001$, compared to unbound fraction. Statistical significance was calculated using Student's unpaired t-test.

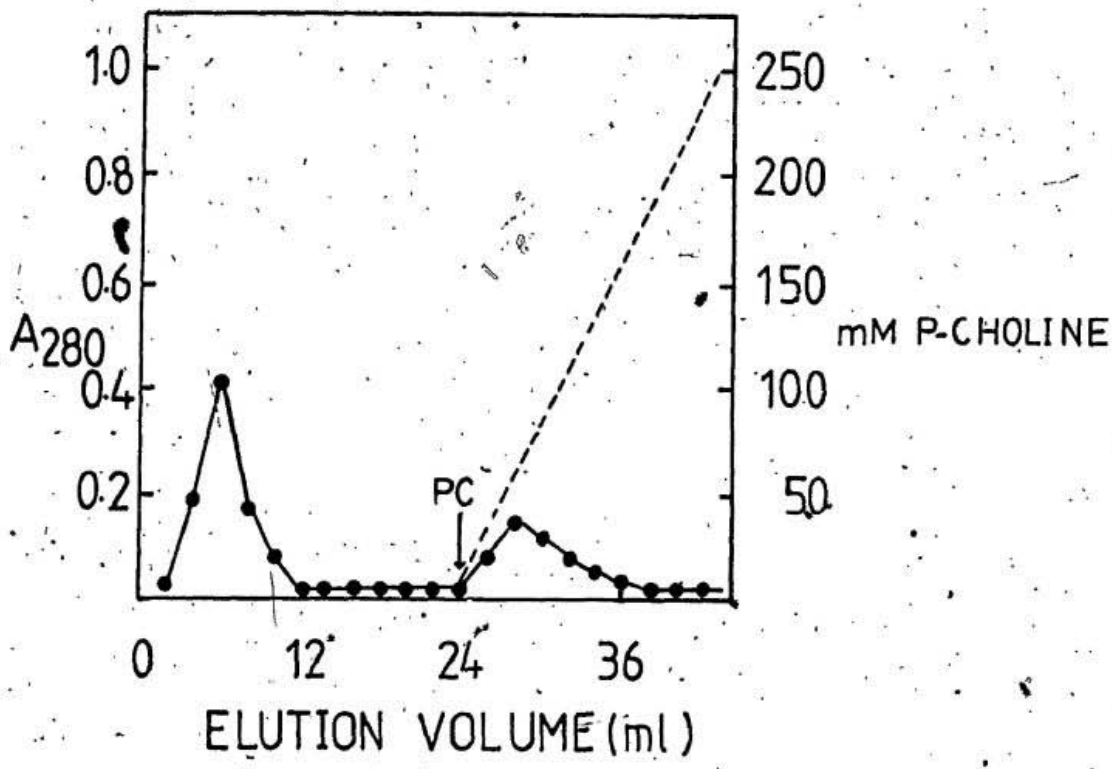


Fig. 37. Elution profile of HDL from Sepharose-PCBP column.

The column and elution conditions were the same as described in fig. 35. The bound HDL was eluted at 68 mM p-choline.

Table 12

Composition of HDL fractions (% by weight) obtained from Sepharose-PCBP column

	applied	unbound	bound
Protein	62.3±6.3	65.9±8.6	54±6.9
Cholesterol	1.50±0.20	0.88±0.13	3.30±1.9
Cholesterol esters	10.0±2.42	8.7±0.96	16.1±2.0
Phospholipids	19.1±3.0	18.5±3.6	24.0±2.5
Triglycerides	1.0±0.86	1.8±0.06	0.8±0.17

The percent lipid of the fractions was calculated from total lipid profiles. Results are presented as the mean ± S.E. (n = 3).

related to the presence of Lp(a) in the bound fraction.

D. Binding of lipoproteins from whole plasma to Sepharose-PCBP.

The results presented above with purified lipoproteins showed that the fractions bound to immobilised PCBP by the application of VLDL and HDL had higher concentrations of apo B and apo E. The possibility that Sepharose-PCBP selectively interacts with these two apoproteins was further investigated by the application of whole normal human plasma (2 ml, 130 mg protein) to the column. A P-choline gradient was then applied and resulted in elution of a bound fraction. Further elution with 1.4M NaCl did not elute any additional protein (Fig. 38). The results in Table 13 show the recovery of lipid and apoproteins in the bound fraction. Apo B (18% of applied) and apo E (8% of applied) were present in the bound fraction. In addition, this fraction contained 6% of cholesterol, 16% of cholesterol esters, 8% phospholipids and 15% of triglycerides.

The percent composition of the bound fraction is presented in Table 14. The chemical composition of this fraction differs from that of typical VLDL or LDL, and may represent the presence of a mixture of lipoproteins.

The fraction eluted by P-choline gradient was delipidated and resolved into three peaks by gel filtration using HPLC (Fig. 38c). The three peaks were identified as apo B, E and C based on their retention times which corresponded to those

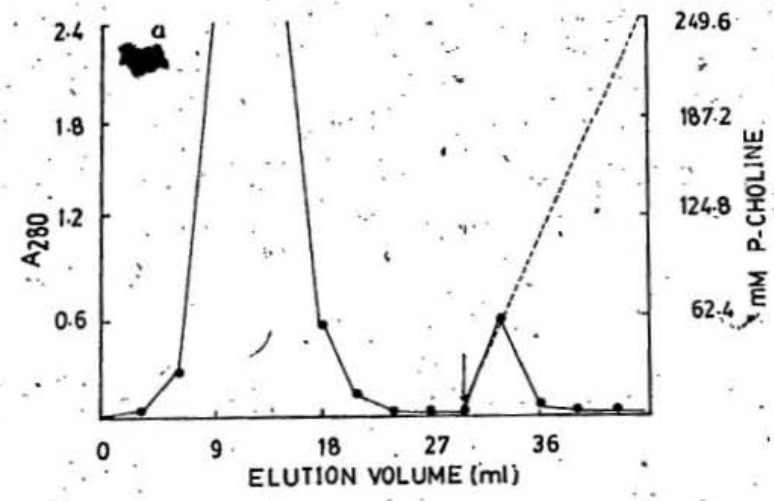
Fig. 38. Elution Profile of Plasma on Sepharose-PCBP and the HPLC resolution of apo B, E and C from plasma fraction bound to Sepharose-PCBP.

a). Elution profile of plasma (2 ml, 130 mg protein)) on Sepharose-PCBP. The bound fraction was eluted by a P-choline gradient (0-250 mM).

b) Elution profile of VLDL apolipoproteins (about 50 g protein; injection volume 50 µl). Eluting buffer was 0.5M sodium sulfate, 0.2 M sodium phosphate (pH 6.8) containing 0.1% SDS. The retention times as indicated on each peak, for Apo B, Apo E and Apo C were 5.08, 6.8 and 8.2 min respectively. The arrows indicate the elution position of molecular weight standards run separately under identical column conditions. The following standards were used 1) thyroglobulin (670,000 MW) 2) ovalbumin (44,000 MW), 3) myoglobin (17,000 MW) and 4) cynocobalamin (1350 MW).

c) Elution profile of apoproteins in fraction bound to Sepharose-PCBP (50µg protein, injection volume, 50 µl). The elution conditions were exactly as in Fig. 38b. The retention times of the three peaks were 5.1, 6.9 and 8.4 min which corresponded to the retention times obtained for Apo B, Apo E and Apo C respectively as shown in Fig. 38b. The retention time of 2-mercaptoethanol in Fig. 38b and c was 13 min. Other details of the HPLC run are as described under experimental procedure. Identification of the apoproteins was based on their designated molecular weights. Absorbance unit full scale (AUFS) in Fig. 38b and C was 0.32.

153A


a
2.4
1.8
1.2
0.6
A280
249.6
187.2
124.8
62.4
mM P-CHOLINE
0
9
18
27
36
ELUTION VOLUME (ml)

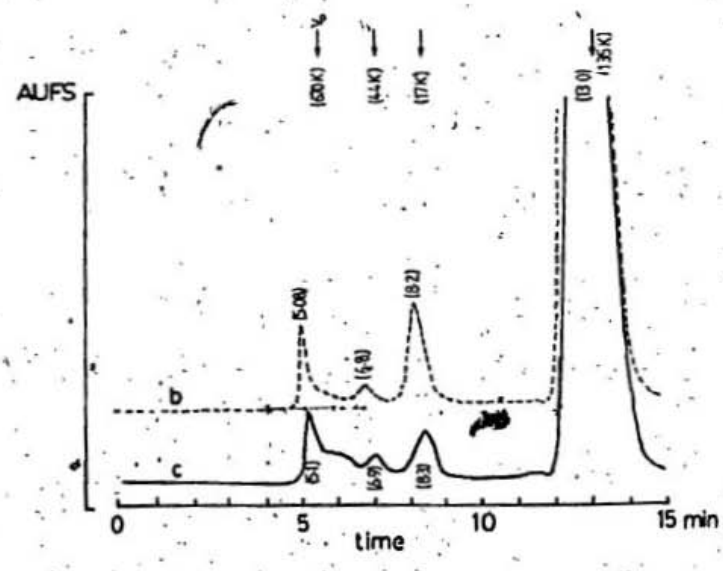

AUFS
(60X)
(44X)
(17X)
(13.0)
(135X)
b
c
0
5
10
15 min
time

Table 13

Binding of lipoproteins from normal human plasma to Sepharose-PCBP.

	applied (mg)	% in bound fraction
Apo B	(0.87-1.62)	18.1±2.9
Apo E	(0.002-0.08)	8.3±1.7
Cholesterol	(0.52-1.06)	6.4±0.9
Cholesterol ester	(3.10-5.14)	16.3±6.0
Phosholipids	(2.41-3.81)	8.1±4.1
Triglycerides	(0.82-2.06)	15.4±5.7

Results are presented as the mean ± S.E. values (n = 3). Apo B and E were quantitated by electroimmunoassay and lipids were determined by gas liquid chromatography.

Table 14

Comparison of the composition (% by weight) of the fraction bound to Sepharose-PCBP from normal human plasma with VLDL and LDL.

	Fraction bound to Sepharose-PCBP	VLDL[a]	LDL[a]
Protein	18.2±4.7	8	20.5
Cholesterol	7.9±1.1	7	8.5
Cholesterol esters	33.5±8.6	12	42.3
Phospholipid	20.1±2.3	18	23.6
Triglyceride	18.2±8.9	55	5.1

The percent composition of the fractions bound from plasma was calculated from total lipid profiles. Results are the means ± S.E. values (n=3).

[a]Adapted from Dolphin (4).

of apo B, E and C from purified VLDL (Fig. 38b). These results demonstrate a binding specificity by Sepharose-PCBP for apo B and E containing lipoproteins even from whole plasma.

E. Role of phosphorylcholine binding site of PCBP and the sialic acid residues on PCBP in the binding of LDL.

To investigate the role of phosphorylcholine binding site on PCBP in the binding of LDL the following experiment was performed. The Sepharose-PCBP (5 ml bed volume) column was pre-equilibrated with excess (35 ml) P-choline (250 mM). Subsequently the column was equilibrated with 50 ml of 0.01M Tris-HCl buffer (pH 7.4) containing 0.15M NaCl and 10 mM Ca^{2+}. Purified LDL (960 μg protein) was then applied to this column. Ninety percent of apo B applied was recovered in the unbound fraction and there was no detectable protein eluted by the P-choline gradient or 1.5M NaCl.

In another experiment the role of sialic acid residues of PCBP in the binding of LDL was examined. To a Sepharose-desialylated PCBP column, LDL (500μg protein) was applied in the presence of Ca^{2+}. The applied LDL bound quantitatively and could be eluted by a P-choline gradient.

F. Effect of chemical modification of apo B on the binding of LDL to Sepharose-PCBP.

The role of lysine and arginine residues of apo B in the binding of LDL to Sepharose-PCBP was investigated. The lysine residues were modified with diketene and the arginine residues with 1,2-cyclohexanedione respectively. It has been previously shown that these reagents are selective for lysine and arginine residues of apo B in LDL (26,27). Furthermore the modification procedures are mild and do not affect the phospholipid content of LDL (26,27).

The modified LDL (500 µg protein) was applied to a Sepharose PCBP in the presence of Ca^{2+} and the bound fraction eluted with a P-choline gradient. Data presented in Table 15 shows that the modification of either 31% or 46% of the total lysine residues resulted in quantitative binding of the modified LDL to Sepharose-PCBP. In contrast, modification of 37% or 40% of arginine residues by 1,2-cyclohexanedione decreased the binding of LDL to Sepharose-PCBP by as much as 68-70% (Table 15).

To test whether the phosphorylcholine binding site on PCBP was involved in the binding of LDL containing modified lysine modified, the Sepharose-PCBP column was equilibrated with P-choline as described before and LDL with modified lysine (500 µg protein, 31% of lysine residues modified) was applied to the column in the presence of Ca^{2+}. The modified lipoprotein was quantitatively recovered in the unbound fraction.

Table 15

Effect of chemical modification of LDL on binding to Sepharose-PCBP

Modification reagent	Residue modified	Percent of total residues modified[a]	Percent bound[b]
Diketene			
Control	None	0	100
1.29µmol	Lysine	31	100
4µmol	Lysine	46	100
Cyclohexanedione			
Control	None	0	100
2h	Arginine	37	32
3h	Arginine	40	30

a) Quantitation of the extent of lysine or arginine modification was based on the difference between lysine or arginine content in the modified versus the control LDL determined by amino acid analysis.

b) LDL (500 µg protein) was applied to Sepharose-PCBP in each experiment and the protein estimated in unbound/bound fractions by the method of Kashyap et al. (141).

5.3. Discussion

The data presented in this chapter demonstrate that PCBP immobilized on Sepharose binds human plasma lipoproteins in the presence of Ca^{2+}. On the basis of current data, it appears that Sepharose-PCBP can selectively bind lipoproteins containing apo B and apo E. This conclusion is supported by several lines of evidence. First, in the HDL binding study, the bulk of Apo A-I (the predominant apoprotein of HDL) was not bound to the column. In contrast almost all of the apo E and Lp(a) lipoprotein (an apo B containing lipoprotein) present in HDL were retained by the column (Table 11). Second, even the passage of whole plasma through the column resulted in the retention of apo B and apo E (Table 13). Third, in the study with VLDL, there was considerable enrichment of apo B and apo E in the bound fraction (Table 7). Finally, all the apo B in the LDL was bound to Sepharose-PCBP column. These findings clearly suggest that PCBP has a special affinity for apo B and apo E containing lipoproteins.

The fact that certain apoproteins interact preferentially with PCBP would suggest that the specific amino acid residues or the amino acid sequence of the apoproteins are involved in the binding. Chemical modification studies undertaken to determine the role of arginine residues of apo B in the binding of LDL to Sepharose-PCBP do indeed support this suggestion. Modification of arginine residues in apo B markedly reduced the binding of LDL to PCBP-Sepharose (Table

15). These results not only establish the importance of apo B but also demonstrate the significance of arginine residues in the binding of LDL to Sepharose-PCBP column. The simplest explanation for the involvement of arginine residues may be that basic group of arginine interacts with the binding site for a cationic quanternary ammonium group on PCBP.

In contrast to the importance of arginine residues, an absence of a role of lysine residues on apo B in the binding is apparent from the studies with lysine modified LDL (Table 15). The binding of LDL to Sepharose-PCBP was unaffected by lysine modification. It may be pertinent to add here that Dicamelli et al (96) have studied the interactions of polycations (eg. poly L-arginine and poly-L-lysine) with human CRP (reviewed in Chapter 1). These studies provided evidence for a polycation binding site on the CRP molecule and suggested that this site has a greater affinity for poly-L-arginine than for polymers of L-lysine.

Investigations on the involvement of the Ca^{2+}-dependent-phosphorylcholine binding site and the sialic acid residues on PCBP in the binding of lipoproteins have yielded interesting results. The results show that the binding of lipoproteins to Sepharose-PCBP had a strict requirement for Ca^{2+} (Table 9). In addition, the experiments with equilibration of Sepharose-PCBP by P-choline suggest that the binding of lipoproteins probably occurs through the phosphorylcholine binding site on PCBP. However, results with Sepharose-desialylated PCBP

indicate an absence of a direct role for the sialic acid residues in the binding of liposomes. Desialylated PCBP immobilized on Sepharose maintained its ability to bind LDL. This result throws further light on the critical role that the sialic acid residues on PCBP play in the inhibition of heparin-lipoprotein precipitation reaction (Chapter 3). It is now apparent that the nature of the two interactions viz. the inhibition of heparin-lipoprotein precipitation and the binding of lipoproteins to PCBP, is different.

It is of interest to note that PCBP specifically binds to apo B and apo E containing lipoproteins, and these are the two apoproteins recognized by LDL receptor on liver and extra-hepatic organs (reviewed in Chapter 1). However whereas chemical modification of both lysine and arginine residues in apo B results in the loss of receptor binding ability, the present studies have shown that only the modification of arginine residues affects the binding to PCBP.

It is well recognized that VLDL are heterogeneous with respect to both size and composition (172,173). Furthermore it has been demonstrated that the various sub-classes of VLDL differ in their ability to interact with the LDL receptors on normal human fibroblasts (174,175). For example, normally the large-sized VLDL though they contain apo B and apo E do not bind to LDL receptors present on normal human. Only the lipolysed and smaller VLDL ($VLDL_3$) are bound and internalised by the LDL receptor pathway (174,175). It was suggested that

both apo B and apo E in the larger VLDL do not possess the appropriate conformation for receptor recognition (174,176). Upon lipolysis, apo B acquires the ability to interact with the LDL receptor. In the present study application of VLDL to Sepharose-PCBP resulted in an unbound and bound fraction (Fig. 35). The unbound fraction also contained some apo B and apo E, albeit less than the bound fraction (Table 7). The unbound fraction was not the result of saturation of the column (Table 7). It is possible that the bound fraction represents a lipolysed VLDL. In support of this, is the data that the triglyceride and apo C II content of the bound fraction of VLDL was lower than the unbound VLDL but apo B and E content were increased (Table 7). Trezzi et al. (172) have fractionated normal human VLDL using heparin-Sepharose chromatography into four subclasses, each increasing in apo B and apo E content with decrease in triglyceride and apo C content. It is therefore likely that in the present studies, Sepharose-PCBP binds to some of the sub-classes of VLDL.

There are several reports in older literature of association between human CRP and plasma lipids.

As far back as 1941, MacLeod and Avery (177) reported that CRP present in human serum and in pathologic fluids was associated with phosphorous-containing substances of lipid character. Wood et al. (178) in their attempts to crystallize human CRP demonstrated the need for delipidation of CRP preparation prior to crystallization, thus suggesting an

association with lipids. Later, Wood (179) used antiserum directed against normal human beta lipoprotein to remove associated lipid containing material in CRP preparations. Sato and Hara (180) characterized the lipids associated with CRP and showed the presence of cholesterol, cholesterol esters and triglycerides.

More recently, several laboratories have been able to demonstrate interactions between human and rabbit CRP with serum lipoproteins but variable results have been obtained by different laboratories.

Pontet et al. (181) have reported that isolated rabbit CRP forms in vitro complexes. Furthermore, it was suggested that rabbit CRP in acute phase serum exists in a complex with LDL.

On the other hand the results of Cabana et al. (182) suggest that the interaction of rabbit CRP is primarily with rabbit VLDL, but not with LDL or HDL. CRP in acute phase serum was shown to be associated with VLDL. This in vitro interaction, demonstrated using immunoelectrophoresis, did not occur in the presence of EDTA or P-choline. These results suggested that the interaction of rabbit CRP with VLDL was Ca^{2+} dependent and probably involved the phosphorylcholine binding site.

Rowe et al. (183) have however, shown that rabbit CRP immobilized on Sepharose binds VLDL as well as LDL from whole normal serum in the presence of Ca^{2+}. In addition,

their studies showed that purified rabbit CRP forms complexes with purified VLDL, LDL as well as β-VLDL, an abnormal lipoprotein present in hypercholesterolemic serum. However, it was shown that in acute phase serum, rabbit CRP was not associated with any serum lipoproteins but in hypercholesterolemic serum part or all of the CRP was found to be complexed with β-VLDL as when analyzed by gel-filtration or immunoelectro-phoresis techniques (183).

Human CRP has also been shown to bind human LDL and trace amounts of VLDL from human plasma in the presence of Ca^{2+} (184). However, this binding was demonstrated only when CRP was immobilized on Sepharose. In fact expression of binding depended upon the concentration of CRP immobilized on Sepharose, occurring appreciably when greater than 1 mg of CRP was coupled per ml of Sepharose. Even under such conditions HDL did not bind to CRP-Sepharose columns. Although the basis for specificity for LDL and VLDL was not investigated, it was speculated that apo B may play part in the interaction of lipoproteins with CRP-Sepharose. In contrast to the immobilized form, free human CRP failed to interact with any normal lipoproteins but formed soluble complexes with β-VLDL in the presence of Ca^{2+} (185).

The various studies mentioned above demonstrate the ability of rabbit and human CRP to interact with lipoproteins. Furthermore, the interaction required the presence of Ca^{2+} and was inhibited by P-choline. However, in view of the

variable results obtained by different laboratories it is difficult to identify a specificity of CRP towards particular class(es) of lipoprotein.

Chapter 6

STUDIES ON THE INTERACTIONS BETWEEN PCBP, LDL AND LDL-RECEPTORS.

6.1 Introduction

The studies described so far in this thesis demonstrated the ability of PCBP to bind to plasma lipoproteins. Furthermore, the binding appears to be selective towards apo B and apo E containing lipoproteins. PCBP immobilised on Sepharose can recognise apo B or apo E in VLDL, LDL or HDL. It may be recalled (Chapter 1) that these apoproteins (apo B-100 and E) are responsible for the specificity of binding certain lipoproteins to cell surface LDL receptors (apo B, E receptor). It is possible that PCBP may bind to apo B or E containing lipoproteins and consequently modulate the interaction of lipoprotein with the LDL receptor. This hypothesis was investigated in a model system consisting of rat liver membrane LDL receptors and human LDL.

Administration of pharmacological doses of 17 α-ethinyl estradiol to rats, has been reported to induce a LDL receptors in the livers (137). These LDL receptors have been characterized using liver membrane preparations. The receptors showed a marked preference for binding apo B and apo E containing lipoproteins, required calcium, and were sensitive to pronase (137). These properties of the membrane receptors are characteristic of the LDL receptors present on normal human fibroblasts (21). The LDL receptor in liver membranes from estradiol-treated rats has been solubilised (186). It was

suggested that this receptor may function to bind apo B and apo E containing lipoproteins (137).

In this chapter in vitro interactions between estradiol treated rat liver membrane LDL receptors, purified human LDL and PCBP are described. An understanding of these interactions with model systems is expected to aid in the evaluation of the biological function of PCBP.

Besides the LDL receptors, at least two other lipoprotein receptors i.e. an apo-E specific chylomicron remanent receptor (187) and an apo A-I or HDL receptor (188) have been described on rat liver. The former recognises only lipoproteins containing apo E and the latter binds lipoproteins containing Apo AI. Since the specific objective of the present study was to determine the effect of PCBP on lipoprotein-LDL receptors, human LDL, containing exclusively apo B, has been utilised. The use of human LDL should minimise the participation of the apo E and apo A-I specific receptors and simplify interpretation of the results.

6.2. Results

A. Binding of ^{125}I-LDL to liver membranes from estradiol treated rats and the effect of PCBP on the binding.

The results of the binding of ^{125}I-LDL to rat liver membrane is shown in Fig. 39a. In the absence of EDTA, the progression of binding was non-linear suggesting the presence of saturable binding sites on the membranes. In the presence

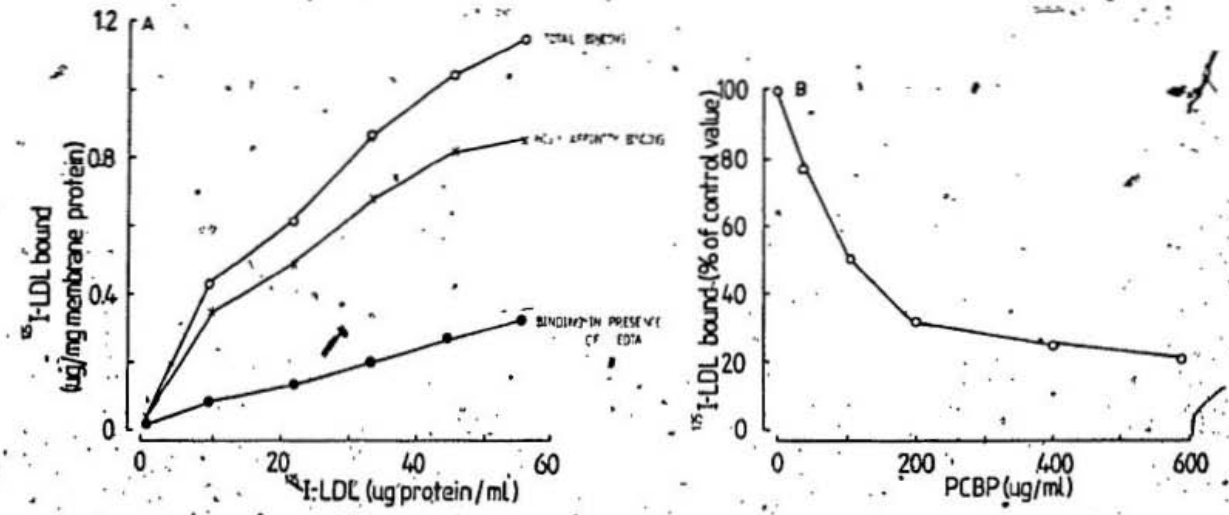


Fig. 39. Binding of ^{125}I-LDL to liver membranes (A) and effect of PCBP on the binding (B):

A) Liver membranes (100,000xg pellet) were prepared from rats treated with 17 α-ethinyl estradiol for 7 days. The binding assays containing 100µg membrane protein and indicated concentration of ^{125}I-LDL (124 dpm/ng protein) were incubated at 0°C for 90 min either in absence (○) or presence (●) of 20 mM EDTA. The ^{125}I-LDL bound to the membrane sediments was determined. High affinity binding was calculated by subtracting the ^{125}I-bound in the presence of EDTA from that bound in the absence of EDTA.

B) Liver membranes (100µg protein) were incubated with, ^{125}I-LDL = 34µg protein/ml , (Specific activity = 124 dpm/ng protein) and increasing concentrations of PCBP and the amounts of ^{125}I-LDL bound to the membranes determined. The 100% value for ^{125}I-LDL bound in the absence of PCBP was 0.85 µg/mg membrane protein. Each data point represents the mean of duplicate assays.

of EDTA, the overall binding of ^{125}I-LDL was reduced as expected (137), although the progression of binding was linear. The difference between binding of ^{125}I-LDL in the absence and presence of EDTA gives a measure of the specific high affinity binding as shown in Fig. 39a. The effect of addition of increasing concentrations of PCBP on the binding of ^{125}I-LDL to estradiol treated rat liver membranes was studied (Fig. 39b). In absence of EDTA, the binding of ^{125}I-LDL to liver membranes decreased in the presence of increasing concentrations of PCBP in a concentration dependent manner.

B. Effect of preincubation of liver membranes with PCBP on the binding of ^{125}I-LDL.

In an attempt to determine whether PCBP binds to liver membrane and thus inhibit the binding of ^{125}I-LDL, liver membranes were preincubated with various concentrations of PCBP. After preincubation, unbound PCBP was removed, ^{125}I-LDL added and the binding of membranes measured. Results presented in Fig. 40a show that preincubation did not affect the binding of ^{125}I-LDL, even though some PCBP (<10%) was bound to the membranes (Fig. 40b) as determined from parallel incubations using ^{125}I-PCBP.

C. Binding of ^{125}I-PCBP to LDL.

Chromatography of a mixture of LDL and ^{125}I-PCBP on Sephacryl S-400 column in the presence of Ca^{2+} (Fig. 41b)

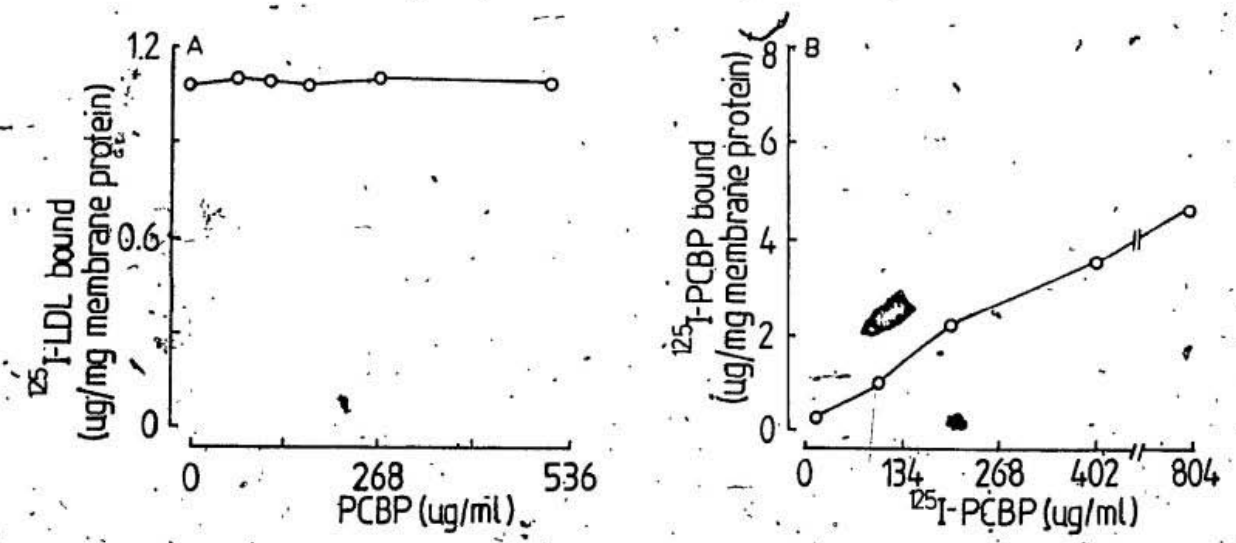


Fig. 40. Effect of preincubation with PCBP on the binding of ^{125}I-LDl to liver membranes (A) and the binding of ^{125}I-PCBP to liver membranes (B).

A) Liver membranes (120 µg protein) were preincubated at 0°C for 90 min with increasing concentrations of PCBP as indicated, followed by the addition of ^{125}I-LDL (48 µg protein/ml, 172 dpm/ng protein) and further incubated for 90 min at 0°C. The amount of ^{125}I-LDL bound to the membrane sediments was determined after centrifugation at 92,000xg for 30 min.

B) Incubation mixture in buffer A contained liver membranes (120 µg protein), ^{125}I-PCBP (1µg) diluted with unlabelled PCBP to give concentrations as indicated. After incubation at 0°C for 90 min. the tubes were centrifuged and ^{125}I-PCBP bound to membranes was determined from the radioactivity associated with the membrane sediments. Each data point represents the mean of duplicate assays.

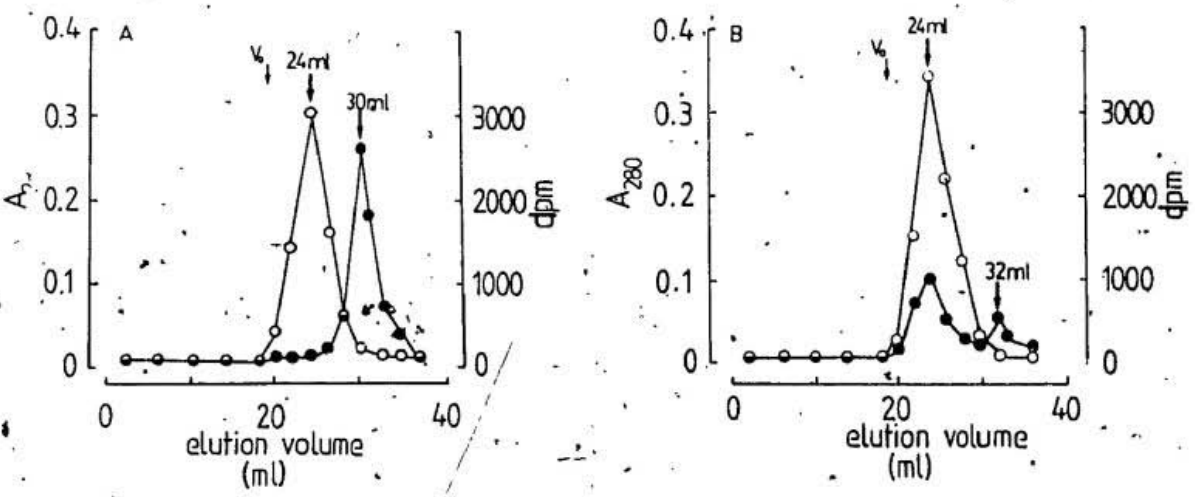


Fig. 41. Gel-filtration of LDL and ^{125}I-PCBP on Sephacryl S400 column after incubation either in absence (A) or presence of Ca^{2+} (B).

A) LDL (0.6 mg protein), ^{125}I-PCBP (0.5µg) diluted with 36.5 µg unlabelled PCBP were incubated at 0°C for 90 min in 0.01M Tris-HCl buffer (pH 7.4) containing 100 mM NaCl in a total volume of 0.2 ml. The mixture was applied on a Sephacryl S400 column (1.5x45 cm) and eluted with 0.01M Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, at a flow rate of 24 ml/h.

B) LDL and ^{125}I-PCBP were incubated and chromatographed exactly as described in A except that the incubation and elution buffers contained 5 mM Ca^{2+}. The recovery of LDL was 90% and that of ^{125}I-PCBP was 80%. Void volume (Vo) was determined using Blue Dextran 2000. (O) denotes the elution of LDL and (-●-) of ^{125}I-PCBP. The results are typical of three such experiments.

resulted in co-elution of ^{125}I-PCBP with LDL. Similar experiments were performed in the absence of Ca^{2+} to ascertain whether the co elution of ^{125}I-PCBP with LDL is Ca^{2+} dependent. As shown in Fig. 41a in the absence of Ca^{2+} the two peaks eluted separately. These findings support the possibility of binding of LDL and PCBP in fluid phase in the presence of Ca^{2+}.

The possibility that the inhibition of the binding of ^{125}I-LDL to the liver membranes by PCBP may be due to the formation of an LDL-PCBP complex was examined by analysing aliquot's of the supernatant from the binding assays by gel-filtration on Sephacryl-400. Incubation of increasing concentrations of ^{125}I-PCBP with LDL and liver membranes, resulted in increasing amounts of ^{125}I-PCBP being complexed in the supernatant with LDL (Fig 42).

During the course of these experiments it was found that the elution volume of free ^{125}I-PCBP in the presence of Ca^{2+} was larger than in the absence of Ca^{2+}. This slightly 'retarded' elution position of ^{125}I-PCBP in the presence of Ca^{2+} may be due to a weak affinity of PCBP for Sephacryl. Sephacryl is prepared by covalently cross-linking alkyl dextran with N,N'-methylene bisacrylamide. A small number of carboxyl groups may also be present. PCBP may interact with alkyl dextran in the presence of Ca^{2+}. In this respect it is relevant to note that in the presence of Ca^{2+}, human CRP has been shown to have a low affinity binding towards

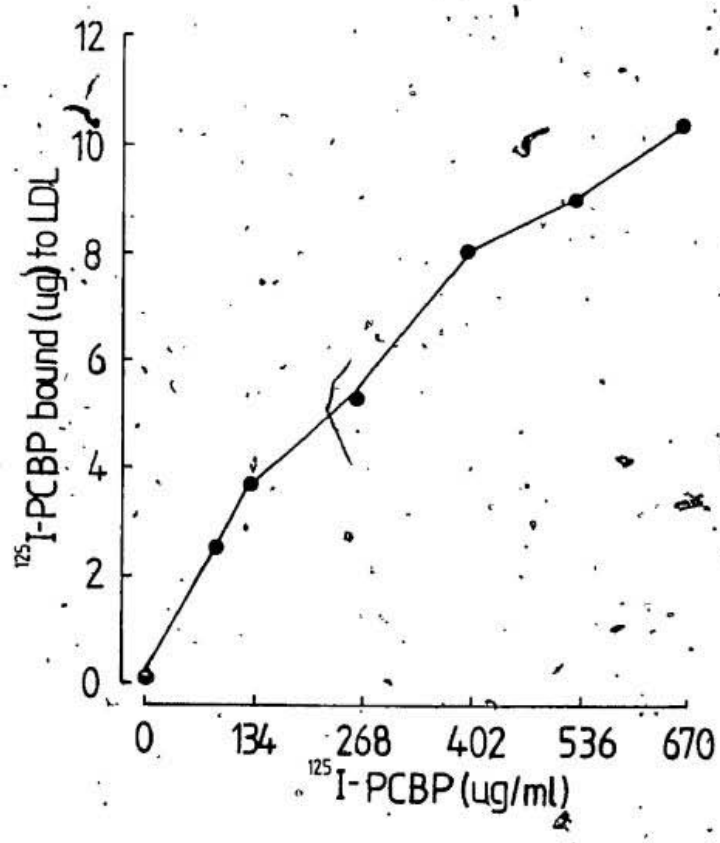


Fig. 42. Detection of LDL-PCBP complex in supernatant from the binding assays by gel-filtration analysis. Binding assays containing liver membranes (100 µg protein), LDL (33.9µg protein/ml), ^{125}I-PCBP (1 µg) diluted with unlabelled PCBP to give final concentrations as indicated were, incubated in buffer A (containing 2.5 mg/ml BSA instead of 20 mg/ml BSA) for 90 min at 0°C. The assay mixtures were centrifuged at 12,800xg for 30 min and the supernatants (100 µl) applied on a Sephacryl S400 column equilibrated with 0.01M Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 5 mM Ca^{2+}. ^{125}I-PCBP complexed with LDL in the supernatants was determined from the radioactivity co-eluting with LDL. Column size and flow rate were as described in Fig. 41. The results are typical of three such experiments.

galactosyl residues in agarose and this binding was prevented by P-choline (189).

6.3. Discussion

The demonstration of selective binding of apo B and apo E containing lipoproteins to Sepharose-PCBP in the last chapter prompted the current investigation on the interaction between PCBP, LDL and LDL receptors. An assay system with liver membranes from estradiol-treated rats as described by Kovanen et al. (137) has been utilised to study the effect of PCBP on ^{125}I-LDL and LDL receptor interactions. The data demonstrated that PCBP inhibits the binding of ^{125}I-LDL to its receptors on liver membranes in a concentration dependent manner (Fig. 39b). Prior incubation of membranes with PCBP did not inhibit the binding when ^{125}I-LDL was subsequently added, suggesting that the LDL-receptor sites on membranes were not blocked by PCBP, although some PCBP was bound to the membranes (Fig. 40a and b). Using Sephacryl S-400 gel filtration chromatography, it was demonstrated that ^{125}I-PCBP co-eluted with LDL in the presence of Ca^{2+} (Fig. 41b). A Ca^{2+} dependent-binding of LDL to PCBP immobilised Sepharose was also demonstrated in chapter 5. Furthermore this binding of ^{125}I-PCBP to LDL was also demonstrable in the LDL-receptor assay (Fig. 42). Incubation of increasing amounts of ^{125}I-PCBP with LDL progressively increased LDL-PCBP complex formation in the supernatant from the assays (Fig. 42). From these

results it is reasonable to conclude that the inhibition of the binding of LDL to the receptors on liver membranes by PCBP resulted from a fluid phase interaction between LDL and PCBP and not due to any binding to receptor sites by PCBP.

Previous work by Goldstein et al. (69,190) has shown that heparin and other sulfated glycosaminoglycans that bind to LDL inhibit the binding of ^{125}I-LDL to its receptors on normal fibroblasts. In addition, Brown et al. (191) have shown that certain positively charged proteins are capable of inhibiting LDL-receptor interactions. It was suggested that these proteins inhibit the binding by binding either to the receptors or to an adjacent site. This is in contrast to the mechanism of inhibition by PCBP which probably involves the binding of LDL to PCBP. The same authors also found that several glycoproteins did not inhibit the binding which gives further credence to the inhibition by PCBP which is a glycoprotein.

In conclusion, the present observations support a notion that an interaction of PCBP with lipoproteins in vivo, could have important implications on the receptor-mediated uptake of lipoproteins.

Chapter 7

REMOVAL OF PLASMA VLDL AND LDL BY PLASMAPHERESIS USING Sepharose-PCBP.

7.1. Introduction

Plasma LDL has been recognized as a primary risk factor in the development of atherosclerosis. Familial hypercholestrolemia is an inherited metabolic disorder, characterized by a genetic deficiency of functional LDL receptors. Because of this deficiency, patients with this disease are unable to clear LDL by LDL-receptor mediated endocytosis (reviewed in Chapter 1) and so have several-fold higher plasma LDL levels compared to normal. These high levels of circulating LDL result in premature onset of atherosclerotic arterial lesions often leading to death (22).

Several studies have been reported (192,193,194) that were designed to reduce plasma LDL cholesterol concentrations in experimental animals and patients with familiar hypercholesterolemia. These studies involved plasmapheresis and extracorporeal removal techniques. Plasmapheresis is typically performed by shunting the blood from arterial branch between the common carotid artery and internal jugular vein, separating the blood into a cell concentrate and a plasma fraction by means of a selective plasma separator membrane. The plasma fraction is passed over an affinity-adsorbent column specific for LDL, and the LDL-depleted plasma recycled into the cell concentrate and returned to the animal through the jugular

vein. The affinity adsorbents that have been utilized are heparin-Sepharose column (192), anti-LDL Sepharose immunoadsorbent column (193) and dextran sulfate-cellulose beads (194).

On the basis of the ability of Sepharose-PCBP column to specifically bind apo B containing lipoproteins (VLDL and LDL) (Chapter 5) which carry the bulk of plasma cholesterol in humans and rabbits the present study was undertaken to evaluate the extent to which plasma VLDL and LDL can be bound by a Sepharose-PCBP column in a plasmapheretic system in vivo. Before proceeding to studies in vivo, experiments were performed in vitro to test whether plasma VLDL and LDL can be removed by a Sepharose-PCBP column. For the experiments in vitro, plasma from diet-induced hypercholesterolemic rabbits was used and the experiments in vivo were performed in normal and diet-induced hypercholesterolemic rabbits.

Diet-induced hypercholesterolemic rabbits were used in this study because of the extreme sensitivity of this animal species to dietary cholesterol. After the initiation of a high cholesterol diet, the plasma cholesterol level rises more than 10-fold accompanied by marked changes in plasma lipoproteins (39,195). The plasma concentrations of VLDL, IDL and LDL rapidly increase (195). In comparison with normal VLDL, in which triglyceride are the predominant lipid and apo B is the major protein, the VLDL particles of cholesterol-fed rabbits contain cholesteryl ester as the major lipid and

are markedly enriched in apo E (39). The hypercholesterolemic VLDL are also larger in size than normal VLDL. Because these VLDL show an abnormal β mobility on electrophoresis, they are called β-VLDL.

In addition to the occurrence of β-VLDL, another alteration in the lipoprotein pattern induced by cholesterol feeding is the appearance of HDL_C, a cholesteryl ester rich lipoprotein that contains apo E as the major apoprotein (39).

Since in rabbits plasma cholesterol levels increase in a very short time after the initiation of high cholesterol diet, this animal model has been used to determine the effectiveness of Sepharose-PCBP columns to bind VLDL and LDL from hypercholesterolemic plasma.

7.2. Results

A. Experiments In vitro

i. Binding of plasma lipoproteins to Sepharose-PCBP as a function of Ca^{2+} concentration.

Studies presented in Chapter 5 had shown a requirement of Ca^{2+} in the binding of lipoproteins to Sepharose-PCBP columns. In order to determine the optimal amount of Ca^{2+} required to bind lipoproteins from hypercholesterolemic plasma, the binding was performed as a funtion of Ca^{2+} concentration. Hypercholestrolemic plasma (2 ml, 29.5 mg cholesterol) was applied to Sepharose-PCBP columns (2 ml bed volume) in the presence of increasing concentrations of

Ca^{2+}. The bound fraction was eluted by EDTA and analysed for total cholesterol, VLDL + LDL cholesterol, HDL cholesterol, triglyceride and total protein content. As shown in Table 16, the amounts of cholesterol, triglyceride and protein bound increased upto a Ca^{2+} concentration of 2.5 mM (Table 16) after which the binding remained unchanged. The distribution of cholesterol in VLDL + LDL and HDL subfractions is shown in Table 16. The results show that >90% of cholesterol bound to Sepharose-PCBP was present as VLDL + LDL cholesterol.

These experiments show that the presence of 2.5 mM Ca^{2+} is sufficient for optimal binding of plasma lipoproteins by Sepharose PCBP columns and that most of the bound cholesterol was recovered in VLDL + LDL subfractions.

Trial experiments had shown that the elution of lipoproteins bound to Sepharose-PCBP with either 20 mM EDTA or 100 mM P-choline resulted in identical recoveries. Therefore 20 mM EDTA was routinely used to elute the bound fractions in all experiments.

ii. <u>Binding of plasma lipoproteins to Sepharose-PCBP as a function of the amount of plasma cholesterol applied</u>.

The binding of lipoproteins from hypercholesterolemic plasma was studied as a function of plasma cholesterol applied, to determine the capacity of Sepharose-PCBP for binding lipoprotein cholesterol.

Increasing volumes of hypercholestrolemic plasma

Table 16

Effect of Ca^{2+} concentration on the in vitro binding of plasma lipoproteins to Sephârose-PCBP

Ca^{2+}[a] concentration (mM)	Total[b] cholesterol bound (mg)	% of bound[c] cholesterol recovered in VLDL + LDL	% of bound cholesterol recovered in HDL	Triglyceride in bound fraction (mg)	Protein in bound fraction (mg)
0	0.14	98	2	0.06	0.03
0.30	3.53	97	3	0.50	1.53
0.60	4.40	96	4	0.6r	1.61
1.25	5.98	94	6	0.72	1.94
2.50	6.10	96	4	0.68	2.43
5.00	6.08	90	10	0.76	2.32
10.0	6.00	92	8	0.61	2.41

a. Hypercholesterolemic plasma (2 ml, mg cholesterol) was applied to Sepharose-PCBP column (2 ml bed volume) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and the indicated concentrations of Ca^{2+}. Prior to application on the column, Ca^{2+} was also added to the plasma at concentrations identical to that of the equilibration buffer. The Sepharose-PCBP columns were operated at 25°C at a flow rate of 20 ml/h and 0.5 ml fractions were collected. Lipoproteins bound from the plasma to Sepharose-PCBP were eluted by 20 mM EDTA.

b. Estimations of total cholesterol and triglyceride in the bound fraction were performed enzymatically on a Hitachi 705 Random axis analyser using the Boehringer-Mannheim automated analysis system. Protein content in bound fraction was estimated following a modified method of Lowry et al. (141).

c. The amount of VLDL + LDL cholesterol present in the bound fraction were calculated from the difference between total cholesterol present in the bound fraction and the amount of HDL cholesterol in the supernatant after Mn^{2+}/heparin precipitation of VLDL + LDL (152) as described in Chapter 2.

(cholesterol concentration, 14.75 mg/ml) were applied to Sepharose-PCBP columns (2 ml bed volume) in the presence of 2.5 mM Ca^{2+}. The lipoprotein bound to the columns was eluted by EDTA and the bound fractions from each experiment analysed for total cholesterol, VLDL + LDL cholesterol, HDL cholesterol, triglyceride and protein. The amount of lipoprotein bound to Sepharose-PCBP increased up to the application of 29 mg plasma cholesterol, after which binding remained unchanged suggesting saturation of the column (Table 17). Most (>90%) of the cholesterol bound to Sepharose-PCBP columns was recovered in VLDL + LDL fractions, thus suggesting that the column mainly binds VLDL + LDL and some (<10%) HDL. The maximum capacity of Sepharose-PCBP columns estimated from these experiments was 1.6 mg cholesterol/ml of Sepharose-PCBP.

iii. Reusability of Sepharose-PCBP column

In order to determine the reusability of Sepharose-PCBP columns, the following experiment was performed:

To a Sepharose-PCBP column (2 ml bed volume), hypercholesterolemic plasma (2 ml, 29.5 mg cholesterol) was applied in the presence of 2.5 mM Ca^{2+}. The bound lipoprotein was eluted with EDTA. After elution with EDTA, the column was equilibrated with several bed volumes of 0.01M Tris-HCl buffer (pH 7.4), containing 0.15M NaCl and 2.5 mM Ca^{2+} and hypercholesterolemic plasma applied again. This experiment was repeated 8 times using the same Sepharose-PCBP column

Table 17

Effect of plasma cholesterol on the in vitro binding of plasma lipoproteins to Sepharose-PCBP

Amount of plasma cholesterol applied (mg)	Total cholesterol bound (mg)	% of bound cholesterol recovered in VLDL + LDL	% of bound cholesterol recovered in HDL	Triglyceride bound (mg)	Protein bound (mg)
2	0.46	91	8	0.09	0.20
3.5	0.57	97	3	0.11	0.24
7.5	1.60	92	8	0.19	0.84
29	6.20	90	10	0.78	2.61
44	5.86	96	4	0.62	2.21
73	5.96	94	6	0.68	2.68

In order to determine the capacity of Sepharose-PCBP column to bind lipoprotein cholesterol, increasing volume of hypercholestrolemic plasma was applied to Sepharose-PCBP columns (2 ml bed volume) in the presence of 2.5 mM Ca^{2+}. The other column conditions were as described in the legend to Table 16. The bound lipoprotein fraction was eluted by 20 mM EDTA. Total cholesterol, VLDL + LDL and HDL cholesterol, triglyceride and protein present in the bound fraction were estimated as described in legend to Table 16.

and under these conditions, no appreciable loss of lipoprotein binding capacity of the column was observed, as judged by the binding of similar amounts of protein and total cholesterol in each experiment. The results suggest that Sepharose-PCBP can be regenerated and used for at least eight experiments.

B. In vivo experiments

Sepharose-PCBP columns were used for experimental plasmapheresis in normal and hypercholesterolemic rabbits as described in Chapter 2. Table 18 summarizes the results of four experiments performed. After each experiment the Sepharose-PCBP column was eluted with EDTA. In each case, the bound fraction showed the presence of cholesterol, triglyceride and protein. Furthermore, as shown in Table 18, most of the cholesterol (> 95 %) bound to the Sepharose-PCBP columns was recovered in the VLDL + LDL fraction and the rest in the HDL fraction. The amount of cholesterol bound to Sepharose-PCBP ranged from 0.22% to 7.7% of the total plasma cholesterol. As shown in Table 18, the amount of cholesterol bound was proportional to the bed volume of the Sepharose-PCBP columns used.

In one experiment (animal number 4), the specificity of the column was evaluated by estimating the recoveries of albumin and total protein. The recoveries were calculated by comparing the pre- and post-plasmapheresis plasma concentrations of albumin and total protein. The post-

Table 18

Binding of plasma lipoproteins to Sepharose-PCBP columns during plasmapheresis experiments.

Rabbit number	Type of rabbit	Total plasma cholesterol (mg/dl)	Bed volume of sepharose-PCBP column used (ml)	Total cholesterol bound (mg)	% of bound cholesterol recovered in VLDL + LDL	% of bound cholesterol recovered in HDL	Triglyceride bound (mg)	Protein bound (mg)	%of total* plasma cholesterol bound
1	normal	13.8	10	3.86	97	3	0.68	0.99	7.7
2	normal	17.7	10	4.2	95	5	0.20	2.21	6.5
3	Hyper cholestrolemic	1782	20	11.62	97	3	1.42	4.2	0.22
4	Hyper cholestrolemic	282	40	27.62	98	2	2.4	14.56	3.27

For plasmapheresis studies, an extracorporeal circuit which included a Sepharose-PCBP column and a plasma separator was constructed. Blood was withdrawn from the carotid artery of the rabbits and plasma separated from the blood cells by passing through the plasma separator. The plasma was then passed through sepharose-PCBP column (bed volume as indicated). After passing through this column, plasma was recombined with blood cell-rich portion and reinfused into the rabbit through the jugular vein. The blood was continuously circulated through the entire circuitary of the plasmapheresis system. After lipoproteins bound to Sepharose-PCBP were eluted with 20 mM EDTA. Total cholesterol, VLDL + LDL and HDL cholesterol, triglyceride and protein in the bound fraction were estimated as described in legend to Table 16. *The total plasma volume of the animal was calculated as described (196).

treatment values were corrected for the dilution during the experiment. Results showed that 94 and 92% of albumin and total protein, respectively, were recovered, thus suggesting that the procedure does not cause a significant degree of non-specific removal of plasma proteins. The small loss may be due to the non-specific binding of fibrin or gammaglobulins to the surface of the equipment used. Such non-specific binding of these plasma proteins to plasmapheresis devices has been reported before (194), although adsorption of small amounts of other components of plasma to Sepharose-PCBP columns cannot be ruled out.

7.3 Discussion

Experiments, *in vitro* and *in vivo*, demonstrate that Sepharose-PCBP columns can retain plasma lipoproteins. Of potential importance is the observation that the Sepharose-PCBP columns bind mainly VLDL + LDL and only some HDL (Table 16,17,18). The VLDL + LDL and HDL fractions in the material bound to Sepharose-PCBP were separated using the heparin/manganese precipitation procedure (152). Heparin/manganese reagent specifically precipitates VLDL + LDL leaving HDL in the supernatant. A possibility exists that the HDL_C (containing apo E as major apoprotein) present in hypercholestrolemic plasma, may bind to Sepharose-PCBP. HDL_C, if present in the fraction bound to Sepharose-PCBP may be precipitated by heparin/manganese reagent. However,

heparin/manganese concentrations utilised in the present study have been shown not to precipitate HDL containing apo E (152). HDL is thought to play an important role in plasma cholesterol transport and, in contrast to LDL, it seems to protect against the progression of atherosclerosis (34). Therefore, the minimal binding of HDL to Sepharose-PCBP columns observed in experiments *in vitro* and *in vivo*, is a desirable property for the potential use of such columns to selectively reduce plasma LDL levels. It may be relevant to add that periodic removal of a small fraction of HDL during plasmapheresis treatments has actually been suggested to induce an ~~el~~evation of plasma HDL concentrations (197).

The Sepharose-PCBP columns do not appear to significantly remove any other plasma proteins as suggested by the almost quantitative (>92%) recoveries of albumin and total protein. However, the column capacity for binding cholesterol in vivo does not appear to be similar to that ~~expected~~ from studies *in vitro*. From the studies *in vitro*, the maximum column capacity was estimated to be 1.6 mg cholesterol per ml of gel but the maximum capacity estimated from the experiments *in vivo*, is only 0.69 mg cholesterol per ml of gel. In comparison with the other affinity adsorbents used in the plasmapheretic lowering of plasma cholesterol, i.e., anti-LDL Sepharose, heparin-agarose and dextran cellulose (192,193,194), the low capacity of Sepharose-PCBP columns in a plasmapheretic system is an obvious disadvantage.

One reason for this reduced column capacity under in vivo conditions may be the haemodynamic and haemodilution factors involved in plasmapheresis. For proper maintenance of the blood pressure and health of the animal through the experiment, the arterial withdrawal rate and the venous infusion rate of the blood were maintained at approximately 22 ml/min withdrawal and 5 ml/min infusion. These rates translate into very high flow rates at which the plasma is chromatographed on Sepharose-PCBP columns. It is possible that at such high flow rates, the Sepharose-PCBP may not function to its capacity. In the experiments in vitro, the Sepharose-PCBP columns were operated at only 20 ml per hour.

Prior to each experiment, the circuitory of the plasmapheresis system was primed with lactated Ringers solution which caused as much as one fold dilution of the total blood volume. This haemodilution will lower the plasma concentration of all the components and may result in the reduced binding capacity of Sepharose-PCBP columns for lipoproteins. Further experiments are required to investigate the influence of these factors on the performance of Sepharose-PCBP columns in vivo.

A possible approach that may circumvent the influence of haemodynamic and haemodilution factors is to use Sepharose-PCBP in a batchwise fashion. Such an approach has been adopted in the use of heparin-agarose for the removal of plasma cholesterol (192). In these studies, blood from patients was

withdrawn into blood transfusion bags containing heparin-agarose and Ca^{2+}. During the withdrawal of blood, the blood bag was mixed by gentle agitation and under these conditions both VLDL and LDL were complexed with heparin-agarose. The blood, deficient of some VLDL and LDL was reinfused into the patients. The simplicity of this procedure makes it possible to attempt similar studies with Sepharose-PCBP.

In the present experiments PCBP was immobilised on Sepharose at a concentration of 1 mg/ml. The possibility exists that increasing the concentration of immobilised PCBP may increase its lipoprotein binding capacity.

In the present experiment it is likely that both VLDL (also β-VLDL in case of hypercholestrolemic rabbits) and LDL are bound by Sepharose-PCBP columns. Although the binding of VLDL is not undesirable, this binding may be at the expense of LDL and therefore reduced amounts of LDL may be removed. In addition in the experiments with hypercholesterolemic rabbits the presence of large quantities of apo E rich β-VLDL may reduce the amount of LDL bound to Sepharose-PCBP.

In their studies with anti LDL-Sepharose, Stoffel and Demant (193) have used plasma separator filters to remove exclusively LDL from the plasma and not VLDL. These plasma separater filters retain the large sized plasma components (MW > 2,000,000) including VLDL, but both LDL and HDL completely pass through these membranes. By using a combination of these

plasma separator filters and the anti-LDL-Sepharose columns in the plasmapheretic system, exclusive removal of LDL from the plasma was achieved. The use of such plasma separator filters may improve the LDL binding capacity of the Sepharose-PCBP columns.

The main aim of this study was to test the ability of Sepharose-PCBP columns to bind VLDL + LDL in a plasmapheretic system. In this respect the pilot study has been successful and generated new ideas to improve the performance of these columns. However, the selectivity of this column must be rigourously tested and its capacity improved before the feasability of use in clinical trials is considered. It may be relevant to add here that the other affinity adsorbents used for lowering plasma LDL levels, have some demerits. For instance, the procedure which utilizes the anti-LDL-Sepharose columns involves, raising monospecific antibodies against LDL, isolation of the antibodies using an appropriate affinity column and then coupling the antibody to a Sepharose. These are time consuming and expensive procedures, requiring a great deal of expertise and manipulations. Potential problems also exist with the use of heparin-Sepharose since numerous important plasma proteins are known to have high affinity to immobilized heparin, e.g., several important components of the coagulation cascade, lipoprotein lipase, and components of complement system (198). Similarly the use of dextran sulfate cellulose has a potential problem considering the

known effects of high molecular weight dextrans to increase precipitation of fibrinogen as well as cause aggregation of platelets. It is possible that Sepharose-PCBP may have yet unidentified advantages over the above mentioned affinity adsorbents. The results obtained from these pilot studies should provide the impetus for further work in this direction.

Chapter 8

PERSPECTIVE AND FUTURE DIRECTIONS

The major purpose of this thesis was to understand the molecular basis of interactions between PCBP and phospholipids and lipoproteins. It was reasonable to expect that insights into the mechanisms of interaction may provide clues as to the function of this protein. Efforts in this direction were made by utilizing conditions of limited complexity. It is likely that under isolated conditions it may be easier to define the properties of PCBP, because of markedly decreased complexity. Once a link with functional capability is identified it would probably be easier to deal with the additional complexities of systems *in vivo*. This approach necessitated the use of model systems and studies *in vitro*. However, such an approach does permit discussions and speculations on the potential implications that may arise from the new information unravelled.

8.1. Possible implications of PCBP-lipoprotein interactions

The results presented in Chapter 3 show a striking difference in the reactivities of glycosylated PCBP and FP and the two non-glycosylated CRP (human and rabbit) towards heparin-lipoprotein interactions. The preservation of phosphorylcholine-binding property in all these proteins suggest that it may be singly important in their biological function(s) of these proteins. However, the results presented

in Chapter 3 show that the presence of sialic acid residues confer an additional ability ie. to inhibit the heparin-lipoprotein interactions. It is tempting to speculate that this inhibitory property of PCBP and FP may have a functional role in the rat and Syrian golden hamster species.

It has been proposed that an injury to the outer endothelial cell lining of the arteries may expose the underlying glycosylaminoglycans (present as proteoglycans), and lead to complexing of plasma VLDL and LDL with the glycosaminoglycans (63). As reviewed in Chapter 1 of this thesis, ionic binding is the most likely mechanism by which plasma lipoproteins can be selectively retained by the subendothelial glycosaminoglycans. This initial entrapment of VLDL and LDL within the vascular endothelium is postulated to be one of the mechanisms associated with the process of lipid accumulation in the arteries.

It is conceivable, therefore, that the presence of PCBP or FP may lead to inhibition of interaction between plasma lipoproteins and arterial glycosaminoglycans. Thus the presence of glycosylated phosphorylcholine binding proteins in the circulation may have a protective role in the deposition of lipoproteins in the artery.

If this line of reasoning is extended further, the failure of non-glycosylated human or rabbit CRP to inhibit heparin-lipoprotein interactions raises the question of whether this inability may have any pathogenetic significance. The results presented in Fig. 13 and 15 show that CRP actually

promotes heparin-lipoprotein interactions. One may therefore suggest that the presence of CRP at a site of arterial endothelial damage, might facilitate the immobilization of VLDL/LDL by glycosaminoglycans.

In this regard it is of interest that recent results of Reynolds and Vance (199) have shown the presence of CRP in human atherosclerotic aorta by immunohistochemical techniques. The pattern of CRP localization was found to be similar to that of apo B. These findings were interpreted to suggest an aggravating role of CRP in the deposition of lipids in the artery.

Thus the contrasting behaviour of PCBP (and FP) and CRP towards heparin-lipoprotein interactions may bear some relationship to the observation that the rat and hamster species are resistant to the lipid deposition in the arteries (200,201), whereas, humans and rabbits are susceptible to such deposition (22,201). In case of Syrian hamster species, given the 100-fold sex difference in serum FP levels, one may expect a corresponding sex difference in lipid deposition. This question remains unanswered at present and complicated by extensive amyloidosis and early demise of female hamsters, both sex limited phenomena, which are possibly related to high FP serum levels (202).

However the suggestion of a role for these proteins in arterial lipid deposition must be supported by further investigation. This becomes especially important in view of

the fact that heparin-lipoprotein interactions were not performed with physiological ionic strength and Ca^{2+} concentrations, although it is generally recognized that atherosclerosis is associated with increased deposition of calcium in the arterial wall (203). Further understanding of the molecular mechanism of lipoprotein-glycosaminoglycan and a role of phosphorylcholine binding proteins in this process may reveal some insights into the mechanism of lipid deposition in the arteries. The Syrian hamster model could be exploited to study the mechanism of lipid deposition after modulating the levels of FP by appropriate hormonal treatments.

Another potential consequence of the inhibition of heparin-lipoprotein precipitation by PCBP and FP involves the enzyme lipoprotein lipase. This enzyme which hydrolyses triglyceride rich lipoproteins occurs on the surface of endothelial cells lining capillary beds and is known to be associated with heparin-like structures that are present on the surface of these cells (204). It has been suggested that the interaction of triglyceride-rich lipoproteins with the heparin structures might serve to anchor the lipoprotein particles to the capillary wall, thus allowing the enzyme access to hydrolyse the triglyceride core (204). Both apo B and apo E present in triglyceride-rich lipoproteins could be involved in the anchoring process. In light of the ability of PCBP and FP to inhibit heparin-lipoprotein interactions it seems reasonable to speculate that PCBP and FP may affect

the lipolytic action of lipoprotein lipase.

The most interesting revelation that emerged from this thesis was the selective interaction of lipoproteins containing apo B and apo E with PCBP immobilized on Sepharose as demonstrated in Chapter 5. When viewed in the overall context of lipoprotein metabolism, several functional roles may emerge from the selective interaction of PCBP with lipoproteins containing apo B and apo E. Theoretically, the binding of PCBP to the surface of lipoproteins may lead to an alteration in the accessibility of the lipoproteins to various enzymes and transfer proteins that participate in lipoprotein metabolism. Such an alteration would be expected to have a profound effect on the structural remodelling that results from the action of the enzymes and transfer proteins. Since the structural remodelling of lipoproteins is an integral component of their metabolism, PCBP could indirectly have an effect on the metabolism of lipoproteins.

In addition, the binding of PCBP to lipoproteins *in vivo* may influence the receptor-mediated uptake of lipoproteins. The results presented in Chapter 7 support the contention that fluid-phase interactions between LDL and PCBP may inhibit the binding of LDL to LDL receptors. In order to postulate a role for PCBP in the process of receptor-mediated uptake of lipoproteins *in vivo* certain unique features of lipid transport in rat should be borne in mind. For instance, relatively little LDL is found in the plasma of rats compared to humans,

while most plasma cholesterol is carried in HDL (39,47). Furthermore, in contrast to human HDL, rat HDL contains significant amounts of apo E (39). In view of the low concentration of LDL in rat plasma, it has been proposed that HDL that contain apo E may be a major vehicle for transport of cholesterol to cells containing LDL receptors (205,206). This role is normally fulfilled by LDL in humans. It is believed that the presence of high amounts of apo E-rich HDL in rat plasma makes it an effective substitute for LDL in this species (205).

Considering these observations in proper perspective, it would appear that apo E-rich HDL is an attractive candidate for interaction with PCBP in rats. It is likely that PCBP-apo E rich HDL interactions would be favored by the presence of large amounts of apo E-rich HDL. The possibility of interaction between PCBP and apo E-containing HDL is in accord with the results presented on Chapter 5 which shows that a subfraction of HDL that contained apo E bound avidly to PCBP immobilized on Sepharose. In addition, preliminary results indicate significant binding of rat HDL to Sepharose-PCBP (211).

What are the consequences of accepting that an interaction between PCBP and lipoprotein in vivo does lead to inhibition of the LDL receptor-mediated uptake? It would suggest that PCBP has function relating to the modulation of transport and distribution of cholesterol to various tissues.

However in view of the total lack of knowledge regarding interactions between PCBP and lipoproteins in vivo these propositions can only be regarded as speculative. The potential implications of interactions between PCBP and lipoproteins do warrant further inquiries along these lines. A good starting point in pursuing this proposal would be to isolate PCBP-lipoprotein complexes (if any) circulating in the plasma and perform physico-chemical characterization of these complexes.

The present studies have also brought to light certain binding properties of Sepharose-PCBP which may prove to be useful in further understanding the metabolism of VLDL and HDL.

The results presented in Chapter 5, show the separation of VLDL into two fractions (unbound and bound) by means of chromatography on Sepharose-PCBP column. The bound fraction was enriched in apo B and apo E compared to the unbound fraction. Similarly, application of HDL to Sepharose-PCBP also resulted in two fractions, where the bound fraction of HDL contained all the apo E and Lp(a) applied.

A number of earlier studies have utilised various techniques to isolate VLDL and HDL subfractions with varying apoprotein content (58,59,172,207,208). These techniques involve ultracentrifugation followed by Geon-Pevikon block electrophoresis. In applying these techniques investigators have used the physical properties of the lipoproteins. In addition repeated ultracentrifugation causes significant

losses of apoproteins, especially apo E, from lipoproteins (38,209). Apo E-enriched subfractions of HDL have also been obtained by heparin/manganese precipitation (70). However, this procedure involves precipitation, washing, resolubilisation, and dialysis of the lipoproteins and may result in the dissociation of apoproteins (210). Heparin-Sepharose affinity chromatography has been used to separate VLDL and HDL subfractions that differ in their ability to bind in vitro with LDL receptors (172,207). The basis for the separation on heparin-Sepharose is the affinity for heparin towards apo B and apo E (56). In this regard one important difference between PCBP binding and heparin binding needs to be pointed out i.e., whereas the neutralization of positive charges on both the lysine or the arginine residues abolishes the ability of lipoproteins to interact with heparin (56), only modification of arginine residues affects the binding of LDL to Sepharose-PCBP. Therefore, the nature of interaction between lipoproteins and heparin appears different from that of PCBP-lipoprotein interactions. On the basis of the results obtained in Chapter 5, it seems possible that subfractionation of VLDL and HDL by Sepharose-PCBP chromatography may provide an alternate approach to the above-mentioned methods. Further studies may be performed to test the ability of the subfractions to interact with LDL receptors on cultured cells. Based on the increased content of apo B and apo E in the bound fractions, it is probable that they

may bind better to the LDL receptors compared to the unbound fractions. If indeed this hypothesis is true, subfractionation by Sepharose-PCBP chromatography may prove to be a useful method to separate and quantitate receptor active and inactive fractions of VLDL and HDL.

8.2. Relationship of PCBP with CRP

While it was not the purpose of this study to establish the relationship of PCBP with CRP, evaluation of its possible relationship may help identify the function of these proteins.

The functions of Pentraxins are poorly understood. Several biological activities in vitro (reviewed in Chapter 1) have been described that are consistent with a role in host defence during injury and inflammation. The induction of CRP by inflammation and injury further supports the concept of a role in host defence during tissue damage and repair. By the same token, the presence of CRP in the normal state may be a disadvantage and therefore the protein is not produced. Failure of CRP to inhibit glycosaminoglycan-lipoprotein interactions in the arteries could be one such disadvantage.

On the other hand, PCBP is present in large amounts in normal state and the current studies show only moderate induction by inflammation (Chapter 3). These observations may be interpreted to suggest that unlike CRP, PCBP may have a definite role in normal daily physiological processes in the rats. A reactivity with plasma lipoproteins and modulation

of their metabolism may be one such role.

The results presented in this thesis also show that PCBP exhibits similarities with human CRP in its ability to bind to phosphatidylcholine containing liposomes as well as plasma lipoproteins. However as discussed above a major difference exists between PCBP and CRP with respect to their effects on heparin-lipoprotein precipitation reaction.

Based on these considerations it appears reasonable to speculate that, even though both the proteins may belong to the same family, there could be functional differences and diversities between PCBP and CRP.

A similar argument may be put forward based on the evolution of PCBP and CRP. In terms of evolution it is possible that both PCBP and CRP may have descended from a common ancestral gene. During the course of evolution there could have been some divergence in the original gene giving rise to divergence in the protein products. Conservation of the phosphorylcholine binding domain may permit the retention of phosphorylcholine binding property, while a divergence in other domains of the protein may result in glycosylation and differential patterns of expression during normal and acute phase states.

The glycosylation and expression during the normal state of the animal may confer additional diverse functions upon PCBP, in comparison to CRP. Further accumulation of information on amino acid sequence, gene structure and the

functions of these proteins will permit a more comprehensive analysis of the relationship between PCBP and CRP.

In summary the results presented in this thesis reveal the ability of PCBP to interact with phosphatidylcholine containing liposomes and human plasma lipoproteins containing apo B and apo E. These interactions require the presence of Ca^{2+} and are probably mediated by a phosphorylcholine binding site on PCBP. The sialic acid residues on PCBP appear not to be directly involved in the binding of plasma lipoproteins. However the sialic acid residues on PCBP are necessary to express its inhibitory effect on heparin-lipoprotein precipitation reaction. The arginine residues on apo B are directly involved in the interaction of LDL with PCBP. Of particular interest is the demonstration *in vitro* that the binding of PCBP to LDL inhibits the LDL-LDL-receptor interactions. This observation may have functional implications *in vivo*.

References

1. Blix, G., Tiselius, A. and Svenson, H. (1941), Lipids and polysaccharides in electrophoretically separated blood serum proteins. J. Biol. Chem. 37, 485-494.

2. Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34, 1343-1353.

3. Bradely, W.A. and Gotto, A.M. (1978) Structure of intact human plasma lipoproteins. In Disturbances in lipid and lipoprotein metabolism. Ed by Dietschy, J.M., Gotto, A.M. and Ontko, J.A. Clinical physiology series, American Physiological Society, Bethesda, MD, pp 117-137.

4. Dolphin, P.J. (1985) Lipoprotein metabolism and the role of apolipoproteins as metabolic programmers. Can. J. Biochem. Cell. Biol. 63, 850-869.

5. Berg, K. (1963) A new serum type in man - the Lp system. Acta Pathol. Microbiol. Scand. 59, 369-382.

6. Alaupovic, P. (1980) The concepts, classification system. In Handbook of Electrophoresis. Ed. by Lewis, L.A. and Opplt. J.J. Vol. 1, pp. 27-46.

7. Kane, J.P., Hardman, D.A. and Paulus, H.E. (1980) Heterogeneity of apolipoprotein B: Isolation of a new species from human chylomicrons. Proc. Natl. Acad. Sci. USA 77, 2465-2469.

8. Kostner, G.M. (1974) Studies of the composition and

structure of human serum lipoproteins. Isolation and partial characterization of apolipoprotein A-III. Biochim. Biophys. Acta 336, 383-395.

9. Mahley, R.W., Innerarity, T.L., Rall, S.C. and Weisgraber, K.H. (1984) Plasma lipoproteins: apolipoprotein structure and function. J. Lipid Res. 25, 1277-1294.

10. Segerest, J.P., Jackson, R.L., Morrisett, J.D. and Gotto, A.M. (1974) A molecular theory of lipid protein interactions in the plasma lipoproteins. Fed. European Biochem. Soc. Letters 38, 247-253.

11. Jackson, R.L., Morrisett, J.D., Gotto, A.M. and Segerest, J.P. (1975) The mechanism of lipid-binding by plasma lipoproteins. Mol. Cell Biochem. 6, 43-50.

12. Assman, G., and Brewer, H.B. (1974) A molecular model of high density lipoprotein. Proc. Natl. Acad. Sci. U.S.A. 71, 1534-1538.

13. Stoffel, W., Zierenberg, O., Tunggal, B.D. and Schreiber, E. (1974) ^{13}C Nuclear Magnetic Resonance Spectroscopic evidence for hydrophobic lipid-protein interactions in human serum high density lipoproteins. Proc. Natl. Acad. Sci. U.S.A. 71, 3696-3700.

14. Shen, B.W., Scanu, A.M. and Kezdy, F.J. (1977) The structures of human serum lipoproteins inferred from compositional analyses. Proc. Natl. Acad. Sci. U.S.A. 74, 837-841.

15. Hamosh, M., and P. Hamosh (1983) Lipoprotein lipase-

its physiological and clinical significance. Mol. Aspects. Med. 6, 199-289.

16. Redgrave, T.G. (1978) Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. J. Clin. Invest. 41, 465-471.

17. Sherrill, B.C., Innerarity, T.L. and Mahley, R.W. (1980) Rapid hepatic clearance of the canine lipoproteins containing only the apo E apoprotein by high affinity receptor. Identity with the chylomicron remanant transport process. J. Biol. Chem. 257, 5646-5655.

18. Weisgraber, K.H., Innerarity, T.L., Rall, S.C. and Mahley, R.W. (1985) Receptor interactions controlling lipoprotein metabolism. Can. J. Biochem. Cell Biol. 63, 898-905.

19. Eisenberg, S. (1980) Plasma lipoprotein interconversions. The origin of low and high density lipoproteins. Ann. N.Y. Acad. Sci. 348, 30-47.

20. Breckenridge, W.C. (1985) The catabolism of very low density lipoproteins. Can. J. Biochem. Cell Biol. 63, 890-987.

21. Goldstein, J.L. and Brown, M.S. (1977) The low density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. 46, 897-930.

22. Brown, M.S. and Goldstein, J.L. (1984) How LDL receptors influence cholesterol and atherosclerosis. Sci. American 251, 58-66.

23. Goldstein, J.L. and Brown, M.S. (1975) Familial hypercholesterolemia: A genetic regulatory defect in cholesterol metabolism. Am. J. Med. 58, 147-150.

24. Goldstein, J.L. and Brown, M.S. (1984) Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J. Lipid Res. 25, 1450-1461.

25. Mahley, R.W. and Innerarity, T.L. (1983) Lipoprotein receptors and cholesterol homeostasis. Biochim. Biophys. Acta 737, 197-227.

26. Mahley, R.W., Innerarity, T.L., Pitas, R.E., Weisgraber, K.H., Brown, J.H. and Gross, E. (1977) Inhibition of lipoprotein binding to cell surface receptors of fibroblasts following selective modification of arginyl residues in arginine-rich and B apoproteins. J. Biol. Chem. 252, 7279-7287.

27. Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1978) Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J. Biol. Chem. 253, 9053-9062.

28. Hamilton, R.L. (1979) Hepatic secretion and metabolism of high-density lipoproteins In Disturbances in lipid and lipoprotein metabolism. Ed. by Dietschy, J.M., Gotto, A.M. and Ontko, J.A. Clinical physiology series, American Physiologica Society, Bethesda, M.D., pp. 155-171.

29. Glomset, J.A. (1968) The plasma lecithin-cholesterol acyltransferase reaction. J. Lipid Res. 9, 155-167.

30. Glomset, J.A. (1979) Lecithin: cholesterol acyl transferase. An exercise in comparative biology. Prog. Biochem. Pharmacol. 15, 41-46.

31. Schaffer, E.J., Eisenberg, S. and Levy, R.J. (1978) Lipoprotein apoprotein metabolism. J. Lipid Res. 19, 667-687.

32. Scanu, A.M., Byrne, R.E. and Mitiovilovic, M. (1982) Functional roles of plasma high density lipoproteins. CRC Crit. Rev. Biochem. 13, 109-140.

33. Steinberg, D. (1978) The rediscovery of high density lipoproteins: a negative risk factor in atherosclerosis. Eur. J. Clin. Invest. 8, 107-109.

34. Miller, G.J. and Miller, N.E. (1975) Plasma high-density lipoprotein concentration and development of ischemic heart disease. Lancet I, 16-19.

35. Tall, A.R. (1986). Plasma lipid transfers proteins. J. Lipid Res. 27, 361-367.

36. Pattnaik, N.M., Montes, A., Hughes, L.B. and Zilversmit, D.B. (1978) Cholesteryl ester exchange protein in human plasma. Isolation and characterisation. Biochim. Biophys. Acta 530, 428-438.

37. Tall, A.R., Forester, L.R. and Bongiovanni, G.L. (1983) Facilitation of phosphatidylcholine transfer into plasma high density lipoproteins by an apoprotein in

the density 1.20-1.26 g/ml fraction of plasma. J. Lipid Res. 24, 277-289.

38. Mahley, R.W. and Holcombe, K.S. (1977) Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. J. Lipid Res. 18, 314-324.

39. Mahley, R.W. (1978) Alterations in plasma lipoproteins induced by cholesterol feeding in animals including man. In Disturbances in lipid and lipoprotein metabolism. Ed. by Dietschy, J.M., Gotto, A.M. and Ontko, J.A. Clinical physiology series, American Physiological Society, Bethesda, MD, pp. 181-197.

40. Mclean, J.W., Fukazawa, C. and Taylor, J.M. (1983) Rat apolipoprotein E mRNA. Cloning and sequencing of double-stranded cDNA. J. Biol. Chem. 258, 8993-9000.

41. Herbert, P.N., Lundmueller, H.G., Bersot, T.P. and Shulman, R.S. (1974) Characterization of rat apolipoproteins. The low molecular weight proteins of rat plasma high density lipoproteins. J. Biol. Chem. 249, 5718-5724.

42. Boguski, M.S., Elshourbagy, N., Taylor, J.M. and Gordon, J.I. (1985) Comparative analysis of repeated sequences in rat apolipoproteins. A-I., A-IV and E. Proc. Natl. Acad. Sci. U.S.A. 82, 992-996.

43. Boguski, M.S., Elshourbagy, N., Taylor, J.M. and Gordon, J.I. (1984) Rat apolipoprotein A-IV contains repetitions of a 22-amino acid segment with amphipathic

helical potential. Proc. Natl. Acad. Sci. U.S.A. 81, 5021-5025.

44. Van't Hooft, F.M., Harman, D.A., Kane, J.P. and Havel, R.J. (1982) Apolipoprotein B (B-48) of rat chylomicrons is not a precursor of the apolipoprotein of low density lipoproteins. Proc. Natl. Acad. Sci. U.S.A. 79, 179-182.

45. Elousen, J., Huang, Y.O., Baker, N. and Kannan Ramaswamy (1981) Apolipoprotein B is structurally and metabolically heterogeneous in the rat. Proc. Natl. Acad. Sci. U.S.A. 78, 157-161.

46. Sparks, C.E., Rader, D.J. and Marsh, J.B. (1983) Metabolism of two forms of apolipoprotein B of VLDL by rat liver. J. Lipid Res. 24, 156-166.

47. Oschery, Y. and Eisenberg, S. (1982) Rat plasma lipoproteins: re-evaluation of a lipoprotein system in animal devoid of cholesteryl ester transfer activities. J. Lipid Res. 23, 1099-1106.

48. Lindhal, U. and Hook, M. (1978) Glycosaminoglycans and their binding to biological macromolecules. Ann. Rev. Biochem. 47, 385-417.

49. Burstein, M., and Samialle, J. (1955) Sur la clarification du serum lipemique par (l heparine in vitro C.R. hebd. Seanc. Acad. Sci. Paris 241, 664-665.

50. Bernfeld, P., Donahue, V.M. and Berkomitz, M.E. (1957) Interaction of human serum β-lipoglobin with polyanions. J. Biol. Chem. 226, 51-64.

51. Burstein, M. and Scholnick, H.R. (1973) Lipoprotein-polyanion-metal interactions. Adv. Lipid Res. 11, 68-108.

52. Bernfeld, P., Nisselbaum, J.S., Berkely, B.J. and Hanson, R.W. (1960). The influence of chemical and physicochemical nature of macromolecular polyanions on their interaction with human serum β-lipoproteins. J. Biol. Chem. 235, 2852-2858.

53. Iverius, P.H. (1972) The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. J. Biol. Chem. 247, 2607-2613.

54. Pan, Y.T., Kruski, A.W., Elbein, A.D. (1978) Binding of heparin to human plasma low density lipoprotein. Arch. Biochem. Biophys. 189, 231-240.

55. Burstein, M. and Legmann, P. (1982) Analysis of serum lipoprotein by precipitation methods. In Lipoprotein Precipitation. Ed. by Clarkson, T.B., Kritchevsky, D. and Pollak, O.J. Monographs on Atherosclerosis, Paris, Vol. 11, pp. 101-129.

56. Mahley, R.W., Weisgraber, K.H. and Innearity, T.L. (1979) Interaction of plasma lipoproteins containing apolipoproteins B and E with heparin and cell surface receptors. Biochim. Biophys. Acta 575, 81-91.

57. Quartfordt, S.H., Jain, R.S., Jakoi, L., Robinson, S. and Shelbourne, F. (1978) The heterogeneity of rat high density lipoproteins. Biochem. Biophys. Res. Commun. 83, 786-793.

58. Marcel, Y.L., Vezina, C., Edmond, D. and Suzue, G. (1980) Heterogeneity of human high density lipoprotein: presence of lipoproteins with and without apo E and their roles as substrates for lecithin: cholesterol acyl transferase reaction. Proc. Natl. Acad. Sci. U.S.A. 77, 2969-2973.

59. Shelbourne, F.A. and Quartfordt, S.H. (1977) The interaction of heparin with an apoprotein of human very low density lipoprotein. J. Clin. Invest. 60, 944-950.

60. Srinivasan, S.R., Radhakrishnamurthy, B., and Berenson, G.S. (1973) Studies on the interaction of heparin with serum lipoproteins in the presence of Ca^{2+}, mg^{2+} and mn^{2+}. Archs. Biochem. Biophys. 170, 334-340.

61. Girard, M.L. and Canal, J. (1968) Conceptions nouvelles sur le role des phospholipids dans la precipitation des lipoproteins seriques par les polysaccharide sulfates. C.V. Hebd. Seanc. Acad. Sci. Paris 266, 842-844.

62. Kim, Y.C. and Nishida, T. (1977) Nature of interaction of dextran sulfate with lecithin dispersions and lysolecithin micelles. J. Biol. Chem. 252, 1243-1249.

63. Radhakrishnamurthy, B., Srinivasan, S.R. and Berenson, G.S. (1982) Glycosaminoglycans in Atherosclerosis. In The Glycoconjugates Vol. IV. Ed. by Horowitz, M.I. pp. 265-300, Academic Press.

64. Camejo, G. (1982) The interaction of lipids and lipoproteins with the intracellular matrix of arterial tissue: its

possible role in atherogenesis. Adv. Lipid Res. 19, 1-53.

65. Brown, M.S. and Goldstein, J.L. (1983) Lipoprotein metabolism in macrophage: Implications for cholesterol deposition in atherosclerosis. Ann. Rev. Biochem. 52, 223-261.

66. Falcone, D.J., Mated, N., Helen, S., Minick, C.R. and Fowler, B.D. (1984) Lipoprotein-heparin-fibronectin-denatured collagen complexes enhance cholesteryl ester accumulation in macrophages. J. Cell. Biol. 99, 1266-1274.

67. Day, C.E., Powell, J.R. and Levy, R.S. (1975) Sulfated polysaccharide inhibition of aortic uptake of low density lipoproteins. Artery 1, 126-137.

68. Weisgraber, K.H., Rall, S.C., Mahley, R.W., Milne, R.W., Marcel, Y.L. and Sparrow, J.T. (1986) Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. J. Biol. Chem. 261, 2068-2076.

69. Goldstein, J.L., Basu, S.K., Brunshede, G.Y. and Brown, M.S. (1976) Release of low density lipoprotein from its cell surface receptors by sulfated glycosaminoglycans. Cell. 7, 85-95.

70. Mahley, R.W. and Innerarity, T.L. (1977) Interaction of canine and swine lipoproteins with the low density lipoprotein receptor of fibroblasts as correlated with heparin/manganese precipitation. J. Biol. Chem. 252, 3980-3986.

71. Kushner, I., Gewurz, H. and Benson, M.D. (1981) C-

-reactive protein and the acute phase response. J. Lab. Clin. Med. 97, 739-749.

72. Tillet, W.S. and Francis, T. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. J. Exp. Med. 52, 561-571.

73. Abernethy, T.J. and Avery, O.T. (1941) The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients sera and the effect of calcium ion on the flocculation reaction with the C-polysaccharide of the pneumococcus. J. Exp. Med. 73, 173-182.

74. Brundish, D.E. and Baddiley, J. (1986) Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. 110, 573-582.

75. Glaudemans, C.P.J. (1975) The interaction of homogeneous murine myeloma immunoglobulins with polysaccharide antigens. Adv. Carbohydrate Biochem. 31, 313-346.

76. Volanakis, J.E. and Kaplan, M.H. (1971) Specificity of C-reactive proteins for choline phosphate residues of Pneumococcal C-polysaccharide: Proc. Soc. Exp. Biol. Med. 136, 612-614.

77. Gewurz, H., Mold, C., Siegel, J. and Fiedel, B. (1982) C-reactive protein and the acute phase response. Adv. Intern. Med. 27, 345-372.

78. Baltz, M.L., De Beer, F.C., Feinstein, A., Munn, E.A., Milstein, C.P., Fletcher, T.C., March, J.F., Taylor,

J. Bruton, C., Clamp, J.R., Davies, A.J.S. and Pepys, M.B. (1982) Phylogenetic aspects of C-reactive protein and related proteins. Ann. N.Y. Acad. 389, 49-75.

79. Woo, P., Kornberg, J.R. and Whitehead, S. (1985). Characterisation of genomic and complementary DNA sequence of human C-reactive protein, and comparison with the complementary DNA sequence of serum amyloid P. component. J. Biol. Chem. 260, 13384-13388.

80. Mantzouranis, E.C., Dowton, S.B., Whitehead, A.S., Edge, M.D., Burns, G.A.P. and Colten, H.R. (1985) Human serum amyloid P-component: cDNA isolation, complete sequence of pre-serum amyloid P-component and localisation of gene to chromosome 1. J. Biol. Chem. 260, 7752-7756.

81. Pepys, M.B. and Baltz, M. (1983) Acute phase proteins with special reference to C-reactive protein and related proteins (Pentraxins) and serum amyloid A protein. Adv. Immunol. 34, 141-142.

82. Bach, B.A., Gewurz, H. and Osmand, A.P. (1977) C-reactive protein in the rabbit: Isolation, characterisation and binding affinity to phosphorycholine. Immunochemistry, 14, 215-219.

83. Oliviera, E.B., Gotschlich, E.C. and Liu, T.Y. (1979) Primary structure of human C-reactive protein. J. Biol. Chem. 254, 489-502.

84. Wang, C.M., Nguyen, N.Y., Yonaha, K., Robey, F. and Liu, T.Y. (1982) Primary structure of rabbit C-reactive

protein. J. Biol. Chem. 257, 13610-13615.

85. Pepys, M.B., De Beer, F.C., Arilbtein, C.P., March, J.F., Feinstein, A., Butress, N., Clamp, J.R., Taylor, J., Bruton, C. and Fletcher, T.C. (1982) C-reactive protein and serum amyloid P-component in the plaice (Pleuronectes platessa L.), A marine teleost, are homologous with their human counterparts. Biochim. Biophys. Acta 704, 123-133.

86. Robey, F.A., Tanake, T. and Liu, T.L. (1983) Isolation and characterisation of two major serum proteins from the dogfish, Mustelus canis, C-reactive protein and amyloid P-component (1985) J. Biol. Chem. 258, 3889-3894.

87. Robey, F.A. and Liu, T.Y. (1981) Limulin: A C-reactive protein from Limulus polyphemus. J. Biol. Chem. 256, 969-975.

88. Gotschlich, E.C. and Edelman, G.M. (1967) Binding properties and specificity for C-reactive protein. Proc. Natl. Acad. Sci. U.S.A. 57, 706-712.

89. Gotschlich, E.C., Liu, T.Y. and Oliveria, E. (1982) Binding of C-reactive protein to c-carbohydrate and PC-substituted protein. Ann N.Y. Sci. 389, 163-171.

90. Young, N.M. and Williams, R.E. (1978) Comparison of the secondary structures and binding sites of C-reactive protein and the phosphorylcholine binding murine myeloma proteins. J. Immunol. 121, 1893-1898.

91. Oliveira, E.B., Gotschlich, E.C. and Liu, T.Y. (1980)

Comparative studies on the binding properties of human and rabbit C-reactive protein. J. Immunol. 124, 1396-1402.

92. Barnum, S., Narkates, A.J., Suddath, F.L. and Volanakis, J.E. (1982) Comparative studies on the binding specificities of C-reactive protein (CRP) and HOPC 8. Ann. N.Y. Acad. Sci. 389, 431-434.

93. Robey, F.A. and Liu, T.Y. (1983) Synthesis and use of new spin labelled derivatives of phosphorylcholine in a comparative study of humans, dogfish and Limulus C-reactive proteins. J. Biol. Chem. 258, 3895-3900.

94. Kilpatrick, J.M., Kearney, J.F. and Volanakis, J.E. (1982) Demonstration of calcium-induced conformational change(s) in C-reactive protein by using monoclonal antibodies. Mol. Immunol. 19, 1159-1165.

95. Potempa, L.A., Maldonado, B.A., Laurent, P., Zemel, E.S. and Gewurz, H. (1983) Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. Mol. Immunol. 20, 1165-1175.

96. Dicamelli, R., Potempa, L.A., Siegel, J., Sugehira, L., Petras, K. and Gewurz, H. (1980) Binding of reactivity of C-reactive protein for polycations. J. Immunol. 125, 1933-1938.

97. Potempa, L.A., Siegel, J.N. and Gewurz, H. (1981) Binding reactivity of C-reactive proteins for polycations. II. Modulatory effects of calcium and phosphocholine. J.

Immunol. 127, 1509-1514.

98. Potempa, L.A., Siegel, J.N. and Gewurz, H. (1982) C-reactive protein (CRP) reactivity with polycations: modulatory roles for calcium, phosphocholine (PC) and heparin. Ann. N.Y. Acad. Sci. 389, 461-462.

99. Lei, K.J., Liu, T., Zon, G., Soravia, E., Liu, T.Y. and Goldman, N.D. (1985) Genomic DNA sequence for human C-reactive protein. J. Biol. Chem. 260, 13377-13383.

99. Liu, T.Y., Robey, F.A. and Wang, Chih-Mei (1982) Structural studies on C-reactive protein. Ann. N.Y. Acad. Sci. 389, 151-162.

100. Kushner, I. and Kaplan, M.H. (1961) Studies of acute phase protein. I. An immunohistochemical method for the localization of C_x-reactive protein in rabbits. Association with necrosis in local inflammatory lesions. J. Exp. Med. 114, 961-974.

101. Kushner, I., Rakita, L. and Kaplan, M.H. (1963) Studies of acute phase protein. II. Localization of C_x-reactive protein in heart in induced myocardial infarction in rabbits. J. Clin. Invest. 42, 286-292.

102. Kaplan, M.H. and Volankis, J.E. (1974) Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. J. Immunol. 112, 2135-2147.

103. Volanakis, J.E. (1982) Complement activation by C-reactive protein complexes. Ann. N.Y. Acad. Sci. 389, 235-250.

104. Siegel, J., Rent, R. and Gewurz, H. (1974) Interactions of C-reactive proteins with the complement system. I. Protamine-induced consumption of complement in acute phase sera. J. Exp. Med. 140, 631-647.

105. Siegel, J., Osmand, A.P., Wilson, M.F. and Gewurz, H. (1975) Interactions of C-reactive protein with the complement system. II. C-reactive protein-mediated consumption of complement by poly-L-lysine polymers and other polycations. J. Exp. Med. 142, 709-721.

106. Claus, D.R., Siegel, J., Skor, D., Osmand, A.P. and Gewurz, H. (1977) Complement activation by interaction of polyanions and polycations in the presence of C-reactive protein. J. Immunol. 118, 83-87.

107. Richards, R.L., Gewurz, H., Osmand, A.P. and Alving, C.R. (1977) Interactions of C-reactive protein and complement with liposomes. Proc. Natl. Acad. Sci. U.S.A. 74, 5672-5676.

108. Richards, R.L., Gewurz, H., Siegel, J. and Alving, C.R. (1979) Interactions of C-reactive protein and complement with liposomes. II. Influence of membrane composition. J. Immunol. 122, 1185-1189.

109. Mold, C., Rodgers, C.P., Richards, R.L., Alving, C.R. and Gewurz, H. (1981) Interaction of C-reactive protein

with Liposomes. III. Membrane requirements for binding. J. Immunol. 126, 856-860.

110. Osmand, A.P., Mortensen, R.F., Siegel, J. and Gewurz, H. (1975) Interactions of C-reactive protein with the complement system. III. Complement-dependent passive haemolysis initiated by CRP. J. Exp. Med. 112, 1065-1077.

111. Mortensen, R.F., Osmand, A.P., Lint, F. and Gewurz, H. (1976) Interaction of C-reactive protein with lymphocytes and monocytes: complement-dependent adherence and phagocytosis. J. Immunol. 117, 774-781.

112. Mortensen, R.F. and Duszkiewicz, J.A. (1977) Mediation of CRP-dependent phagocytosis through mouse macrophage F_cR-receptors. J. Immunol. 119, 1611-1616.

113. Mold. C., Du Clos, T.W., Nakayama, S., Edwards, K.M. and Gewurz, H. (1982) C-reactive protein reactivity with complement and effects on phagocytosis. Ann. N.Y. Acad. Sci. 389, 251-262.

114. James, K., Baum, L.L., Vetter, M.L. and Gewurz, H. (1982) Interactions of C-reactive protein with lymphoid cells. Ann. N.Y. Acad. Sci. 389, 274-285.

115. Fiedel, B.A. and Gewurz, H. (1976) Effects of C-reactive protein on platelet function. I. Inhibition of platelet aggregation and release reactions. J. Immunol. 116, 1289-1294.

116. Fiedel, B.A., Potempa, L.A., Frenzke, M.E., Simpson, R.M. and Gewurz, H. (1982) Platelet inhibitory effects

of CRP preparations are due to a co-isolating low molecular weight factor. Immunology 45, 15-22.

117. Fiedel, B.A., Simpson, R.M. and Gewurz, H. (1982) Effects of C-reactive protein (CRP) on platelet function. Ann. N.Y. Acad. Sci. 389, 263-273.

118. Fiedel, B.A. and Gewurz, H. (1986) Cleaved forms of C-reactive protein are associated with platelet inhibition. J. Immunol. 136, 2551-2555.

119. Vigo, C. (1985) Effect of C-reactive protein on platelet-activating factor induced platelet aggregation and membrane stabilisation. J. Biol. Chem. 260, 3418-3422.

120. Kilpatrick, J.M. and Virella, G. (1985) Inhibition of platelet-activating factor by rabbit C-reactive protein. Clin. Immunol. Immunopathol. 37, 276-281.

121. Hanahan, D.J. and Nelson, D.R. (1984) Phospholipids as dynamic participants in biological processes. J. Lipid Res. 25, 1528-1535.

122. Robey, F.A., Jones, K.A., Tanaka, T. and Liu, T.Y. (1984) Binding of C-reactive protein to chromatin and nucleosome core particles: A possible physiological role of C-reactive protein. J. Biol. Chem. 259, 7311-7316.

123. Robey, F.A., Jones, K.D. and Steinberg, A.P. (1985) C-reactive protein mediates the solubilisation of nuclear DNA by complement in vitro. J. Exp. Med. 161, 1344-1356.

124. Coe, J.E. (1977) A sex-limited serum protein of Syrian hamsters: definition of female protein and regulation

by testosterone. Proc. Natl. Acad. Sci. U.S.A. 74, 730-733.

125. Coe, J.E., Margossian, S.S., Slayter, H.S. and Sogn, J.A. (1981) Hamster female protein: A new pentraxin structurally and functionally similar to C-reactive protein and amyloid P component. J. Exp. Med. 153, 977-991.

126. Coe, J.E. (1983) Homologs of CRP: A diverse family of proteins with similar structure. In Contemporary Topics in Molecular Immunology. Vol. 9, Ed. by Inman, F.P. and Kindt, T.J. Plenum Publishing Corporation pp. 211-238.

127. Coe, J.E. (1982) Female protein of the Syrian hamster: A homology of C-reactive protein. Ann. N.Y. Acad. Sci. 389, 299-307.

128. Dowton, S.B., Woods, D.E., Mantzouranis, E.C. and Colten, H.R. (1980) Syrian hamster female protein: Analysis of female protein primary structure and gene expression. Science 228, 1206-1208.

129. Mookerjea, S. (1973) Phosphorylcholine: A specific promoter of heparin and serum -lipoprotein interaction. Biochem. Biophys. Res. Comm. 53, 580-587.

130. Mookerjea, S. (1978) Mechanism of heparin and serum lipoprotein interactions: Effects of calcium, phosphorylcholine, and a serum factor. Can. J. Biochem. 56, 746-752.

131. Nagpurkar, A. and Mookerjea, S. (1981) A novel

phosphorylcholine-binding protein from rat serum and its effect on heparin-lipoprotein complex formation in the presence of calcium. J. Biol. Chem. 256, 7440-7448.

132. Nagpurkar, A., Francis, J., Saxena, U. and Mookerjea, S. (1986) Subunit structure composition of rat serum phosphorylcholine binding protein and its significance in binding to phosphorylcholine and serum lipoproteins. Can. Fed. Biol. Sc. Abst. No. 288.

133. Collins, J., Saxena, U., Sarkar, M., Nagpurkar, A., and Mookerjea, S. (1984) Synthesis and secretion of serum phosphorylcholine-binding protein by rat hepatocytes. Biochim. Biophys. Acta 799, 97-101.

134. Pontet, D., D'Asnieres, M., Gache, D., Escaig, J., and Engler, R. (1981) A new pentraxin (serum amyloid P-component) in the rat: Evidence for two quarternary structures and effect of ligands on self-assocation. Biochim. Biophys. Acta 671, 202-210.

135. De Beer, F.C., Baltz, M.L., Munn, E.A., Fienstein, A., Taylor, J., Bruton, C., Clamp, J.R. and Pepys, M.B. (1982) Isolation and characterization of C-reactive protein and serum amyloid P component in the rat. Immunology, 45, 55-69.

136. Taylor, J.A., Bruton, C.J., Anderson, J.K., Mole, J.E., De Beer, F.C., Baltz, M.L. and Pepys, M.B. (1984) Amino acid sequence homology between rat and human C-reactive protein. Biochem. J. 221, 903-906.

137. Kovanen, P.T., Brown, M.S. and Goldstein, J.L. (1979) Increased binding of low density lipoprotein to liver membranes from rats treated with 17 α-ethinyl estradiol. J. Biol. Chem. 254, 11367-11373.

138. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

139. Davis, B.J. (1964) Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121, 404-427.

140. Warren, L. (1959) The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234, 1971-1975.

141. Kashyap, M.L., Hynd, B.A. and Robinson, K. (1980) A rapid and simple method for measurement of total protein in very low density lipoproteins by the Lowry assay. J. Lipid Res. 21, 491-49.

142. O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

143. McFarlane, A.S. (1958) Efficient trace-labelling of proteins with iodine. Nature (Lond). 182, 53.

144. Wong, L. and Rubinstein, D. (1979) Turnover of apo E in normal and hypercholesterolemic rats. Atherosclerosis, 34, 2149-2158.

145. Laurell, C.B. (1972) Electroimmunoassay, Scand. J. Clin. Lab. Invest. 29, 21-37.

146. Dolphin, P.J., Breckenridge, W.C., Dolphin, M.A. and

Tan, M.H. (1984) The lipoproteins of human umblical cord blood apolipoprotein and lipid levels. 51, 109-122.

147. Tam, S.P. and Breckenridge, W.C. (1983) Apolipoprotein and lipid distribution between vesicles and HDL-like particles formed during lipolysis of human very low density lipoproteins by perfused rat heart. J. Lipid Res. 24, 1343-1357.

148. Kuksis, A., Myher, J.J., Marci, L., and Geher, K. (1975) Determination of plasma lipid profiles by automated gas chromatography and computerized data analysis J. Chromatogr. Sci. 13, 423-431.

149. Folch, J., Lees, M. and Sloane Stanely, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

150. Fiske, C.H. and Subba Row, Y. (1925) The colorimetric determination of phoshorus. J. Biol. Chem. 66, 375-400.

151. Bartlett, G.R. (1959) Phosphorous assay in column chromatography. J. Biol. Chem. 234, 466-468.

152. Gibson, J.C., Rubenstein, A. and Brown, W.V. (1984) Precipitation of apo-E containing lipoproteins by precipitation reagents for apolipoproteins B. Clin. Chem. 30, 1784-1788.

153. Camejo, G., Lopez, A., Lopez, F. and Quinones, J. (1985) Interaction of low density lipoproteins with arterial proteoglycans. Atherosclerosis 55, 93-105.

154. Fontaine, M. and Malmendier, C.L. (1978) The effect of

fasting on the interactions between human low density lipoproteins and calcium, heparin and chondroitin sulfate. Clin. Chim. Acta 94, 201-205.

155. Avila, E.M., Lopez, F. and Camejo, G. (1978) Properties of low density lipoproteins related to its interaction with arterial wall components. In vitro and in vivo studies: Artery 4, 36-60.

156. Cabana, V.G., Siegel, J. and Gewurz, H. (1983) Binding of CRP and lipoproteins to heparin-agarose. Fed. Proc. 42, Abst. No. 4381.

157. Baltz, M.L., De Beer, F.C., Feinstein, A. and Pepys, M.B. (1982) Calcium-dependent aggregation of human serum amyloid P component. Biochim. Biophys. Acta 701, 229-236.

158. Hansen, B., Hutchcraft, C. and Gewurz, H. (1982) Interaction of serum amyloid P-component with heparin. Fed. Proc. 41, Abst. No. 1697.

159. Cabana, V.G., Gewurz, H. and Siegel, J.N. (1983) Inflammation-induced changes in rabbit CRP and plasma lipoproteins. J. Immunol. 130, 1736-1742.

160. Mendoza, S.G., Zerpa, A., Carrasco, H. (1983) Estradiol, testosterone, apolipoproteins, lipoprotein cholesterol and lipolytic enzymes in man with premature myocardial infarction and angiographically assessed coronary occlusion. Artery, 12, 1-23.

161. Jackson, R.L., Chan, L., Snow, D.L. and Means, A.R.

(1978) Hormonal regulation of lipoprotein synthesis. In Disturbances in Lipid and lipoprotein metabolism. Ed. by Dietschy, J.M., Gotto, A.M. and Ontko, J.A. Clinical Physiology Service, American Physiological Society, Bethesda, MD, 139-154.

162. Colley, C.M. and Ryman, B.E. (1976) The liposome: from membrane model to therapeutic agent. Trends Biochem. Sci. 1, 203-205.

163. Sparrow, J.T. and Gotto, A.M. (1982) Apolipoprotein/lipid interactions: Studies with synthetic polypeptides. CRC Rev. Biochem. 13, 87-107.

164. Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672.

165. Yeagle, P.L., Hutton, W.C., Huang, C-h. and Martin, R.B. (1977) Phospholipid head-group conformations, intermolecular interactions and cholesterol effects: Biochemistry 16, 4344-4349.

166. Yeagle, P.L., Langdon, R.G. and Martin, R.B. (1977) Phospholipid-protein interaction in human low density lipoprotein detected by ^{31}P-nuclear magnetic resonance. Biochemistry 16, 3487-3491.

167. Yeagle, P.L., Martin, R.B., Pottenger, L. and Langdon, R.G. (1978) Location and interactions of phospholipid and cholesterol in human low density lipoprotein from ^{31}P Nuclear Magnetic Resnance. Biochemistry, 17, 2707-2710.

168. Katz, S.L. and Philips, M.C. (1986) Packing of cholesterol molecules in human low density lipoprotein. Biochemistry 25, 1562-1568.

169. Tsujimoto, M., Inouve, K. and Nojima, Shoshichi (1981) Reactivity of human C-reactive protein with positively charged liposomes. J. Biochem. 90, 1507-1514.

170. Volanakis, J.E. and Wirtz, K.W.A. (1979) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. Nature (Lond). 281, 155-157.

171. Albers, J.J. and Hazzard, W.R. (1974) Immunochemical quantitation of plasma Lp(a) lipoprotein. Lipids 9, 15-26.

172. Trezzi, E., Calvi, C., Roma, P. and Catapano, A.L. (1983) Subfraction of human very low density lipoproteins by heparin-sepharose affinity chromatography. J. Lipid Res. 24, 790-795.

173. Patsch, W., Patsch, J.R., Kostner, G.M., Sailer, S. and Braunsteiner, H. (1978) Isolation of subfractions of human very low density lipoproteins by ultracentrifugation. J. Biol. Chem. 253, 4911-4915.

174. Catapano, A.L., Gianturco, S.H., Kinnunen, P.K.J., Eisenberg, S., Gotto, A.M. and Smith, L.G. (1979). Suppression of 3-hydroxy-3-methylglutaryl-CoA reductase by low density lipoproteins produced in vitro by lipoprotein lipase action on non-suppressive very low density lipoproteins. J. Biol. Chem. 254, 1007-1009.

175. Gianturco, S.H., Packard, C.J., Shepherd, J., Smith, L.C., Catapano, A.L., Sybers, H.D. and Gotto, A.M. (1980) Abnormal suppression of 3-hydroxy-3-methyl glutaryl-CoA reductase activity in cultured human fibroblasts by hypertriglyceridemic very low density lipoprotein subclasses. Lipids, 15, 456-463.

176. Gianturco, S.H., Gotto, A.M., Hwang, S-L.C., Karlin, J.B., Lin, A.H.Y., Prasad, S.C. and Bradley, W.A. (1983) Apolipoprotein E mediates uptake of S_f-100-400 hypertriglyceridemic very low density lipoproteins by the low density lipoprotein receptor pathway in normal human fibroblasts. J. Biol. Chem. 258, 4526-4533.

177. MacLeod, C.M. and Avery, D.T. (1941) The occurrence during acute infections of a protein not normally present in the blood. Isolation and properties of the reactive protein. J. Exp. Med. 73, 183-192.

178. Wood, H.F., McCarty, M. and Slater, R.J. (1954) The occurrence during acute infections of a protein not normally present in blood. V. Physical-chemical characteristics of the protein crystallized by a modified technique. J. Exp. Med. 100: 71-79.

179. Wood, H.F. (1963) Crystallization of C-reactive protein following removal of associated lipid-containing material by antiserum to normal human beta lipoprotein. Yale J. Biol. Med. 36, 241-248.

180. Sato, S. and Hara, J. (1968) Immunochemical studies on

the lipid-protein complex. 1. The lipid containing C-reactive protein. Jpn. J. Exp. Med. 38, 373-

181. Pontet, M., Ayrault-Jarrier, M., Burdin, J., Gelin, M. and Engler, R. (1979) Preparation et forme serique de la proteine reactive C de lapin, Biochimie 61, 1293-1299.

182. Cabana, V.G., Gewurz, H. and Siegel, J.N. (1982) Interaction of very low density lipoproteins (VLDL) with rabbit C-reactive protein. J. Immunol. 128, 2342-2348.

183. Rowe, I.F., Soutar, A.K., Trayner, I.M., Baltz, M.L., De Beer, F.C., Walker, L., Bowyer, D., Herbert, J., Feinstein, A. and Pepys, M.B. (1984) Rabbit and rat C-reactive proteins bind apolipoprotein B-containing lipoproteins. J. Exp. Med. 159, 604-616.

184. De Beer, F.C., Soutar, A.K., Baltz, M.L., Trayner, I.M., Feinstein, A. and Pepys, M.B. (1982) Low density lipoprotein and very low density lipoprotein are selectively bound by aggregated C-reactive protein. J. Exp. Med. 156, 230-242.

185. Rowe, I.F., Soutar, A.K., Trayner, I.M., Thompson, G.R. and Pepys, M.B. (1984) Circulating human C-reactive protein binds very low density lipoproteins. Clin. Exp. Immunol. 58, 237-244.

186. Roach, P.D. and Noel, S.P. (1985) Solubilisation of the 17 -ethinyl estradiol-stimulated low density lipoprotein receptor of male rat liver. J. Lipid Res. 26, 713-720.

187. Cooper, A.D., Erickson, S.K., Nutik, R. and Shrewsburry,

M.A. (1982) Characterisation of chylomicron remanant binding to rat liver membranes. J. Lipid Res. 23, 42-52.

188. Nakai, T., Otto, P.S., Kennedy, D.L. and Whayne, T.F. (1976) Rat high density lipoprotein subfraction (HDL_3) uptake and catabolism by isolated rat liver parenchymal cells. J. Biol. Chem. 251, 4914-4921.

189. Volanakis, J.E. and Narkates, A.J. (1981) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers and complement. J. Immunol. 126, 1820-1826.

190. Goldstein, J.L., and Brown, M.S. (1976) The LDL pathway in human fibroblasts: A receptor-mediated mechanism for the regulation of cholesterol metabolism. Curr. Top. Cell. Regul. 11, 147-181.

191. Brown, M.S., Devel, T.F., Basu, S.K. and Goldstein, J.L. (1978) Inhibition of the binding of low-density lipoprotein to its cell surface receptor in human fibroblasts by positively charged proteins. J. Supramolecular Structure 8, 223-234.

192. Lupien, P.J., Moorjani, S. and Awad, J.C. (1976) A new approach to the management of familial hypercholesterolaemia: Removal of plasma-cholesterol leased on the principle of affinity chromatography. Lancet, June, 1265-1265.

193. Stoffel, W. and Demant, T. (1981) Selective removal of apolipoprotein B-containing serum lipoproteins from blood plasma. Proc. Natl. Acad. Sci. 78, 611-615.

194. Yokoyama, S., Hayashi, R., Kikkawo, T., Tani, N., Takada, S., Hatanaka, K. and Yamamoto, A. (1984) Specific sorbent of apolipoprotein B-containing lipoproteins for plasmapheresis: Characterisation and experimental use in hypercholesterolemic rabbits. Arteriosclerosis 4, 276-282.

195. Kushwaha, R.S. and Hazzard, W.R. (1978) Catabolism of very low density lipoproteins in the rabbit: Effect of changing composition and pool size. Biochim. Biophys. Acta 528, 176-189.

196. Kita, T., Brown, M.S., Bilheimer, D.W. and Goldstein, J.L. (1982) Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. Proc. Natl. Acad. Sci. U.S.A. 79, 5693-5697.

197. Parker, T.S., Gordon, B.R., Saal, S.D., Rubin, A.L. and Ahrens, E.H. (1986) Plasma high density lipoprotein is increased in man when low density lipoprotein (LDL) is lowered by LDL-pheresis. Proc. Natl. Acad. Sci. 83, 777-781.

198. Comper, W.D. (1981) Extracellular interactions in heparin (and related polysaccharides): Structural and functional properties. Ed. by Huglin, M.B. Polymer monographs Vol. 7. Gordon and Breach Science Publishers, pp. 175-237.

199. Reynolds, G.D. and Vance, R.P. (1986) Immunohistochemical

localisation of C-reactive protein in human atherosclerotic aorta. Fed. Proc. 45, Abst. No. 1794.

200. Soret, M.G., Blanks, M.C., Gerritsen, G.C. and Day, C.E. (1975) Diet-induced hypercholestrolemia in diabetic and non-diabetic chinese hamsters. Adv. Exp. Med. Biol. 67, 329-343.

201. Wissler, R.W., Eilert, M.L., Schroeder, M.A. and Cohen, L. (1954) Production of lipomatous and atheromatous arterial lesions in the albino rat. A.M.A. Arch. Pathol. 57, 333-351.

202. Coe, J.E. and Ross, M.J. (1985) Hamster female protein, a sex limited pentraxin, is a constituent of syrian hamster amyloid. J. Clin. Invest. 76, 66-74.

203. Henry, P.D. (1985) Atherosclerosis, calcium and calcium antagonists. Circulation, 72, 456-459.

204. Cryer, A. (1981) Tissue lipoprotein lipase activity and its action in lipoprotein metabolism. Int. J. Biochem. 13, 525-541.

205. Van't Hooft, F. and Havel, R.J. (1982) Metabolism of apolipoprotein E in plasma high density lipoproteins from normal and cholesterol-fed rats. J. Biol. Chem. 257, 10996-11001.

206. Sigurdsson, G., Noel, S.P. and Havel, R.J. (1979) Quantification of the hepatic contribution to the catabolism of high density lipoproteins in rats. J. Lipid Res. 20, 316-324.

207. Weisgraber, K.H. and Mahley, R.W. (1980) Subfractionation of human high density lipoproteins by heparin-sepharose affinity chromatography. J. Lipid Res. 21, 316-325.

208. Huff, M.W. and Telford, D.E. (1984) Characterization and metabolic fate of two very low density lipoprotein subfractions separated by heparin-sepharose chromatography. Biochim. Biophys. Acta 796, 251-256.

209. Fainaru, M., Glangeaud, M.C. and Eisenberg, S. (1975) Radioimmunoassay of human high density lipoprotein apoprotein A-I. Biochim. Biophys. Acta 386, 432-443.

210. Rooke, J.A. and Skinner, E.R. (1979) The dissociation of apolipoproteins from rat plasma lipoproteins during isolation by precipitation with polyanions. Int. J. Biochem. 10, 329-335.

211. Nagpurkar, A., Saxena, U., Dolphin, P. and Mookerjea, S. (1985) The binding of human and rat apo B and E containing lipoproteins to rat C-reactive protein. Proc. 7th International Symposium on Atherosclerosis, Melbourne, Abst. No. 377.