STUDIES ON RAT C-REACTIVE PROTEIN: BINDING TO ISOLATED RAT HEPATOCYTES AND CLEARANCE FROM CIRCULATION

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by

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A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements
for the degree of Master of Science

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ISBN 0-315-65298-5

ABSTRACT

The clearance mechanism of C-reactive proteins (CRP) was examined in this study. *In vitro* binding assays using isolated rat hepatocytes were followed by clearance studies *in vivo* and using perfused rat liver.

It was shown that rat asialo CRP was rapidly cleared from the circulation (half-life about 4 min), whereas rat and rabbit CRP remained in the circulation for much longer periods of time (half-life 7.8 h for rat CRP). The clearance of rat asialo CRP was shown to be mediated through the hepatic asialoglycoprotein receptor, and this clearance was not affected by the phosphorylcholine binding domain of rat asialo CRP. Results of the clearance studies also suggested that, in addition to liver, lungs might also be involved in the clearance of CRP.

It was observed that large amounts of rat asialo CRP, rat CRP and rabbit CRP bound to isolated rat hepatocytes, and the binding was shown to be mediated predominantly through the phosphorylcholine binding domain of these proteins. In addition, rat asialo CRP was shown to bind to the hepatic asialoglycoprotein receptor on the hepatocytes, despite the avid phosphorylcholine binding domain-mediated binding of this protein. It was demonstrated that the binding of large amounts of rat asialo CRP, rat CRP and

rabbit CRP to the hepatocytes through the phosphorylcholine binding domain resulted from a disruption of the hepatocyte surface.

ACKNOWLEDGEMENTS

I am deeply grateful to my supervisors, Dr. S. S. Mookerjea and Dr. A. Nagpurkar, for the opportunity to carry out this study in their laboratory and for their distinguished guidance throughout this project. Their advice has been very inspiring and invaluable. The continued financial support from their grants has made it possible for me to concentrate on scientific and academic subjects. I am especially grateful to Dr. S. Mookerjea for taking every care of me, and to Dr. A. Nagpurkar for critically reading and painstakingly revising this thesis.

I am truly thankful to Dr. K. M. W. Keough for helpful discussion and suggestions. His advice and encouragement have been invaluable.

I wish to express my sincere appreciation to members of Biochemistry Department at Memorial University of Newfoundland for creating a pleasant and stimulating atmosphere. I am especially thankful to everyone in our laboratory for all the help they have given me, and for the friendly, cooperative and pleasant atmosphere they have created.

I wish to thank the School of Graduate Studies in Memorial University of Newfoundland for financial support in the form of a Memorial University Graduate Fellowship. Thanks are also due to the Heart and Stroke Foundation of

Canada and the Medical Research Council of Canada for financial support to this project.

I truly thank my friends for their interest in my progress. I heartily thank Canadian people for their generosity and hospitality. They have helped me adapt myself to a totally different culture. I owe many things that I have learned in various aspects of my life to them.

I am particularly very grateful to my grandmother, my parents and other members of my family for all their love, support and everything they have given me. Finally, I truthfully thank my motherland. I have all my best wishes to her and dedicate this thesis to her.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGP	α_1-acid glycoprotein (bovine)
Apo	Apoprotein
Asialo AGP	Desialylated α_1-acid glycoprotein (bovine)
Rat asialo CRP	Desialylated rat C-reactive protein
Asn	Asparagine
BSA	Bovine serum albumin
CNBr	Cyanogen bromide
CPM	Count per minute
CPS	C-polysaccharide
CRP	C-reactive protein
EDTA	Ethylenediamine-tetraaceticacid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
FP	Syrian hamster female protein
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcNAc	N-acetylglucosamine
HPLC	High performance liquid chromatography
IL-1	Interleukin-1
IL-6	Interleukin-6
Iodo-Gen	1,3,4,6-tetrachloro-3α,6α-diphenylglycouril

LDL	Low density lipoprotein
Man	Mannose
MW	Molecular weight
NANA	N-acetylneuraminic acid
NSB	Non-specific binding
PC (P-choline)	Phosphorylcholine
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PCBP	Phosphorylcholine binding protein
SAP	Serum amyloid P component
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error
TB	Total binding
VLDL	Very low density lipoprotein

LIST OF KEY WORDS

C-reactive protein

Asialoglycoprotein receptor

Hepatocytes

Binding assays

Perfusion

Clearance

Liver

Lung

Phosphorylcholine binding protein

Rat

Rabbit

Chapter 1. INTRODUCTION

C-reactive proteins (CRP) are present in a wide range of species, from horseshoe crab (Limulus polyphemus) to human being. CRP derives its name from a phosphorylcholine-mediated reactivity with the C-polysaccharide of the pneumococcal cell wall (Abernethy and Avery, 1941; MacLeod and Avery, 1941; Volanakis and Kaplan, 1971). These proteins have two common characteristics that have been conserved throughout evolution: i) a pentameric disc-like configuration (Osmand et al., 1977) (with the only exception of horseshoe crab CRP, which has a hexagonal ring-shaped structure (Robey and Liu, 1981)), and ii) a calcium dependent binding to the phosphorylcholine ligand (Volanakis and Kaplan, 1971). In addition, they share extensive amino acid sequence homology (Oliveira et al., 1979; Wang et al., 1982; Pepys et al., 1984; Nguyen et al., 1986a). Based on their glycosylation status, CRP can be divided into two categories: a) Non-glycosylated CRP, such as human and rabbit CRP; and b) Glycosylated CRP, such as rat CRP. CRP are synthesized in the liver by hepatocytes (Kushner et al., 1980; Collins et al., 1984). However, their clearance pathway is not known.

In the 1970's, Ashwell discovered that, after the removal of terminal sialic acids, most mammalian circulatory glyco-proteins are taken up by the liver via the hepatic

asialoglycoprotein receptor (Morell et al., 1971: Ashwell and Harford, 1982; Ashwell, 1984). In the case of the CRP family, since some are glycosylated while others are non-glycosylated, it is appropriate to study if Ashwell's clearance pathway is applicable to glycosylated CRP and if glycosylated and non-glycosylated CRP are cleared through a common mechanism. In addition, it is important to examine if the highly conserved phosphorylcholine binding domain of CRP would affect the binding of asialo CRP to the hepatic asialoglycoprotein receptor, and if this binding would affect their subsequent clearance through Ashwell's pathway.

As a necessary background for this thesis, an overview on C-reactive proteins and an outline of the structure and clearance pathways of glycoproteins have been provided.

Section I. OVERVIEW ON C-REACTIVE PROTEINS

A. DISCOVERY AND DEFINITION OF C-REACTIVE PROTEINS

In 1930, Tillett and Francis (1930) observed that sera obtained from patients during acute febrile illnesses precipitated with an extract of a pneumococcus, which was first designated as Fraction C and subsequently as C-polysaccharide (CPS). The substance responsible for this reactivity was termed C-precipitin. It was later proven to be a protein not normally present in the blood and was designated as C-reactive protein (CRP) (Abernethy and Avery, 1941).

Subsequently, it was demonstrated that the reaction of CRP towards CPS is directed to the phosphorylcholine residue on CPS (Volanakis and Kaplan, 1971). This binding specificity of human CRP has been utilized to define CRP homologues in other species. Proteins from the sera of other species which exhibit a similar Ca^{2+}-dependent binding to immobilized CPS, phosphorylcholine, or phosphorylcholine derivatives are also designated as CRP. These proteins closely resemble human CRP in molecular appearance, subunit composition and amino acid sequence (Bach et al., 1977; Pepys et al., 1978a).

Some members of the CRP family have been referred to by other names. For example, limulus CRP had been known as limulin before it was characterized as a member of the CRP

family (Robey and Liu 1981). When rat CRP was first isolated by Nagpurkar and Mookerjea, it was termed rat phosphorylcholine binding protein (PCBP) on the basis of its property of binding to phosphorylcholine (Nagpurkar and Mookerjea, 1981). It was subsequently shown to be identical to rat CRP independently isolated by Pontet et al. (1981) and de Beer et al. (1982a). In this thesis, the term rat CRP is used, instead of rat PCBP.

B. DISTRIBUTION OF CRP

Since the discovery of CRP in humans, CRP has been found in a variety of species (Table 1). CRP has been conserved throughout the evolution process, from horseshoe crabs to human beings. The molecular appearance, subunit composition, amino acid sequence and binding specificity of CRP have been conserved during this process.

C. ISOLATION AND PROPERTIES OF CRP

1). Isolation of CRP

CRP was first isolated in a crystalline form from a lipoprotein-rich fraction of human serum by precipitating with CPS, followed by delipidation and salt fractionation (McCarty, 1947; Wood et al., 1955). More recent methods of purification involve affinity chromatography techniques using immobilized CPS, phosphorylcholine or phosphorylcholine derivatives (Osmand et al., 1975; Young and

Table 1. **DISTRIBUTION OF C-REACTIVE PROTEINS**

Name	Subunits	Glycosylation	MW
Human CRP[a]	5	No	117.5 kd
Monkey CRP[b]	?	?	?
Bovine CRP[c]	5	No	150 kd
Goat CRP[d]	5	Yes	120 kd
Dog CRP[b]	?	?	?
Rabbit CRP[e, f]	5	No	120 kd
Cat CRP[g]	?	?	?
Rat CRP[h, i]	5	Yes	125 kd
Mouse CRP[j, k]	10?	?	200 kd
Chicken CRP[l]	?	?	21 kd/subunit
Rainbow trout CRP[m]	5?	?	110 kd
Dogfish CRP[n]	10	?	250 kd
Plaice CRP[o]	10	Yes	188 kd
Lumpsucker CRP[p]	10	No?	150 kd
Limulus CRP[q, r]	12	Yes	500 kd

[a]Gotschlich and Edelman (1965); [b]Riley and Coleman (1970)
[c]Maudsley et al., (1987b); [d]Maudsley et al., (1987a)
[e]Robey et al., (1984); [f]Bach et al., (1977)
[g]Pepys and Maudsley (1987); [h]de Beer et al., (1982a)
[i]Nagpurkar and Mookerjea (1981); [j]Oliveira et al., (1980)
[k]Bodmer and Siboo (1977); [l]Pepys et al., (1978a)
[m]Winkelhake and Chang (1982); [n]Robey et al., (1983)
[o]Pepys et al., (1982a); [p]Fletcher et al., (1981)
[q]Robey and Liu (1981); [r]Fernandez-Moran et al., (1968)

Williams, 1978; Volanakis et al., 1978; Nagpurkar and Mookerjea, 1981). Isolation of CRP from large quantities of serum, pleural, or peritoneal fluids can be accomplished by adsorbing CRP to the affinity resin in the presence of calcium and eluting with phosphorylcholine or a calcium-chelating agent.

2). General Physicochemical Properties of CRP

Molecular shape

Typically, CRP comprises 5 identical subunits (Oliveira et al., 1979). When examined under an electron microscope, CRP appears as cyclic pentamers and has therefore been referred to as pentraxins (Osmand et al., 1977). Human, rabbit and rat CRP all have this typical conformation. In dogfish (Robey et al., 1983), plaice (Pepys et al., 1982a), and lumpsucker (Fletcher et al., 1981), CRP is composed of 10 subunits. These subunits either form two pentameric discs interacting face-to-face or consist of 5 dimers which aggregate in a pentameric shape. One exception to the common molecular shape of CRP is horseshoe crab CRP, which assumes a hexagonal ring-shaped structure consisting of 12 subunits (Robey and Liu, 1981).

The subunits in CRP are usually held together non-covalently. However, in rat CRP, three of its five identical subunits are non-covalently held together, while the other two are covalently bonded through a disulphide bridge

(Nagpurkar and Mookerjea, 1981; de Beer et al., 1982a).

Composition

CRP subunits consist of 205-218 amino acids (Gotschlich and Edelman, 1965; Wang et al., 1982; Nguyen and Suzuki, 1986a). The molecular weights of CRP range from 110 to 500 kd (Volanakis et al., 1978). In some species, such as rat (de Beer et al., 1982a; Nagpurkar and Mookerjea, 1981), goat (Maudsley et al., 1987a), plaice (Pepys et al., 1982a), hamster (Coe, 1977) and horseshoe crab (Robey and Liu, 1981), CRP subunits contain carbohydrate chains. In the case of rat CRP, which contains 18% (w/w) carbohydrate (Nagpurkar and Mookerjea, 1981), it has been shown that each of its subunits is glycosylated at position Asn-128 with a complex biantennary oligosaccharide chain (Sambasivam et al., 1990). In human (Gotschlich and Edelman, 1965), rabbit (Robey et al., 1984), cow (Maudsley et al., 1987b), and lumpsucker (Fletcher et al., 1981), CRP does not contain any carbohydrate. Therefore, based on their glycosylation status, CRP can be divided into two classes: glycosylated CRP and non-glycosylated CRP (Table. 1).

Stability

The stability of purified CRP is dependent on temperature, pH, and protein concentration (Macintyre, 1988). Solutions of CRP at concentrations of 50 μg/ml to

1 mg/ml at pH 7-8 are stable for weeks at 4°C and for months at -20°C. Under conditions of lower pH or higher protein concentration, CRP molecules tend to aggregate. In alkaline solutions or with repeated freeze-thawing, dissociation of CRP into subunits or intermediate forms may be appreciable.

3). **Binding Properties of CRP**

Binding of CRP to phosphorylcholine

The ligand that CRP binds best is phosphorylcholine (Volanakis and Kaplan, 1971). Substitution of the phosphate monoester group of phosphorylcholine with other acidic groups markedly decreases or abolishes the binding of CRP (Young and Williams, 1978; Gotschlich, 1982). It has been shown that the interaction of CRP with CPS and many other complex polysaccharides of microbial, fungal, or metazoan parasite origin is mediated through the phosphorylcholine moiety of these polysaccharides (Tomasz, 1967; Pery and Luffau, 1979). The higher affinity for phosphorylcholine compared with other phosphate monoesters suggests that CRP recognizes both the positively charged trimethylammonium group and the negatively charged phosphate group.

Human and rabbit CRP bind 5 molecules of phosphoryl-choline per molecule of protein (Liu et al., 1982). Dogfish CRP binds two phosphorylcholine molecules per subunit dimer (Robey et al., 1983). This suggests one phosphorylcholine binding domain on each subunit of CRP. One exception is rat

CRP, which binds three, rather than five, molecules of phosphorylcholine (Nagpurkar and Mookerjea, 1981).

The binding of phosphorylcholine by CRP requires that CRP first binds calcium, usually in a ratio of one to two Ca^{2+} per CRP subunit (Gotschlich and Edelman, 1965). The conformational change induced by Ca^{2+} makes the phosphorylcholine binding domain of CRP available to the phosphorylcholine ligand (Potempa et al., 1981; Young and Williams, 1978).

The amino acid sequence of CRP has been compared with the known sequences of phosphorylcholine binding myeloma proteins. On the basis of this comparison, it has been proposed that the sequence Phe-Tyr-Met-Glu in CRP may be part of its phosphorylcholine binding domain (Young and Williams, 1978).

Binding of CRP to polycations

In the absence of calcium ions, CRP binds polycations such as poly-L-lysine and poly-L-arginine polymers, lysine-rich and arginine-rich histones, myelin basic protein, and leucocyte cationic protein (Siegel et al., 1974; Potempa et al., 1981). At appropriate concentrations of CRP and ligand, the complexes aggregate and precipitate.

In the absence of phosphorylcholine, calcium inhibits the interaction of CRP and polycations. However, in the presence of phosphorylcholine, calcium promotes rather than

inhibits these interactions (DiCamelli et al., 1980).

4). **Acute Phase Levels of CRP**

CRP in human (Zeller, 1986), monkey (Riley and Coleman, 1970), rabbit (Winkelhake and Chang 1982), chicken (Pepys et al., 1978a), and rainbow trout (Winkelhake and Chang 1982) are typical acute phase proteins. Normal sera in these species contain only trace amounts of CRP. However, during inflammation or tissue damage, CRP concentrations in serum dramatically increase, sometimes up to several thousand-fold over normal values. In these species, CRP is among the first group of proteins that are synthesized at a greatly enhanced rate during acute phase response, and termination of the stimulation is accompanied by the return of their serum concentrations to normal levels.

CRP in rat (Collins et al., 1984; de Beer et al., 1982a) is a moderate acute phase protein. It is present in substantial amount in normal rat serum (0.5-0.6 mg/ml). During acute phase reactions, its serum concentration increases by about two-fold (Collins et al., 1984).

CRP in cow (Maudsley et al., 1987b), goat (Maudsley et al., 1987a), dogfish (Robey et al., 1983), plaice (White et al., 1982), and horseshoe crab (Robey and Liu, 1981) are normal components in the sera. They do not undergo major change in serum concentration following an acute stimulus.

D. BIOSYNTHESIS AND TURNOVER OF CRP

1). Biosynthesis of CRP

Experiments conducted in rabbits (Kushner and Feldman, 1978a) or with rat hepatocytes (Collins et al., 1984) have shown that CRP is synthesized and secreted by the liver. De novo CRP synthesis has been demonstrated using perfused livers (Kushner et al., 1980). CRP has been identified histochemically in the cytoplasm of hepatocytes but not in any other type of hepatic cells of rabbits during inflammation (Kushner and Feldman, 1978a).

2). Increased CRP Synthesis During Acute Phase Response

In species in which CRP is an acute phase protein, the peak level of CRP in serum correlates well with the severity of tissue damage (Kushner et al., 1978b; Aronsen et al., 1972). Following an acute stimulus, increased CRP synthesis starts in the periportal area, and then spreads to involve all cells across the liver lobule (Kushner and Feldmann, 1978a). The peak level depends on the duration of increased CRP production rather than on the initial rate of increase in synthesis (Kushner et al., 1978b). These findings indicate that CRP is produced by progressively increasing numbers of hepatocytes after inflammatory stimulus. They also suggest that a circulating mediator(s), which is released in association with inflammation and necrosis, is responsible for the induction of CRP synthesis. There is

evidence that the circulating mediators may be interleukin-1 (IL-1) and interleukin-6 (IL-6) (Moshage et al., 1988). Both IL-1 and IL-6 have been shown to stimulate the synthesis of CRP by the liver, but IL-6 is believed to play a key role in the process (Moshage et al., 1988).

3). **Other Sites of CRP Biosynthesis**

Although liver is the main source of serum CRP, it has been shown that a subset of peripheral blood lymphocytes are also able to synthesize CRP, which apparently remains bound to the surface of these cells.(Ikuta et al., 1986; Kuta and Baum, 198[illegible]). The biological significance of these findings is not clear at present.

4). **In vivo Clearance of CRP**

The half-life of human CRP in the circulation of normal rabbits is about 4-6 h, and it remains the same during the course of acute phase response (Chelladurai et al., 1983). In vivo turnover studies of CRP have also been carried out in mice and rats (Baltz et al., 1985). The half-life of human CRP in mice and that of rat CRP in rats were also in the range of 4-6 h. The half-lives of these CRP were independent of their circulating levels and were not affected by CPS (Baltz et al., 1985).

E. PROTEINS RELATED TO CRP

1). Serum Amyloid P Component (SAP)

Serum amyloid P component (SAP) was first identified as a component of amyloid deposits in humans (Cathcart et al., 1965). SAP and CRP in humans are similar to each other in the following aspects: i). They both belong to a unique family of proteins called pentraxins. Their subunits are arranged in an annular disc-like configuration with cyclic pentameric symmetry (Osmand et al., 1977); ii). There is about 60% amino acid sequence homology between human CRP and SAP (Osmand et al., 1977; Anderson and Mole, 1982); iii). They both have Ca^{2+}-dependent binding capacity to certain ligands (Pepys and Baltz, 1983; Skinner and Cohen, 1988). However, whereas CRP has a strong Ca^{2+}-dependent binding property to phosphorylcholine, SAP binds to agarose, but not to phosphorylcholine (Pepys et al., 1977a; Pepys et al., 1977b).

In other species, serum proteins which share with human SAP the property of binding to agarose but not to phosphorylcholine have also been designated as SAP (Pepys et al., 1978a). SAP has been found in almost all vertebrate species, including rabbit, rat, mouse, pig, goat, sheep, donkey, human, cow, marine toad, plaice, flounder, and dogfish (Pepys et al., 1978a).

2). **Syrian Hamster Female Protein (FP)**

In Syrian hamsters, a CRP analogue is expressed in high levels only in females (Coe, 1982). Due to its sex-related distribution, this protein is designated as hamster female protein (FP). In normal adult female Syrian hamster, FP is present at high serum levels in the range of 0.7-3.0 mg/ml, while in normal males the level is much less (0.01-0.02 mg/ml).

Like CRP and SAP, FP is composed of five identical subunits and exhibits the pentameric structure (Coe, 1983). FP is a unique protein in that it can bind both phosphorylcholine and agarose in the presence of calcium (Coe, 1983). The amino acid sequence of FP has 69% homology to human SAP and 50% homology to human CRP (Dowton et al., 1980). Although FP binds to phosphorylcholine, it has been shown to be a component of hamster amyloid deposits (Coe, 1983). Therefore, FP appears to be a homologue of both human CRP and SAP.

3). **Other Phosphorylcholine Binding Proteins**

Anti-phosphorylcholine myeloma proteins

Anti-phosphorylcholine myeloma proteins have phosphorylcholine binding property. However, unlike the phosphorylcholine binding of CRP, their binding of phosphorylcholine is independent of Ca^{2+} (Pollet and

Edlhoch, 1973). Compared to CRP, these proteins show much less binding specificity for the phosphate moiety of the ligand (Young and Williams, 1978).

Perforin

Perforin is a pore-forming protein in cytolytic T-lymphocytes. It has been demonstrated that phosphorylcholine acts as a Ca^{2+}-dependent receptor molecule for perforin (Tschopp et al., 1989).

F. BIOLOGICAL ACTIVITIES OF CRP

A number of biological properties of CRP have been reported, based on their interactions with macromolecules and with various types of cells. Most of these properties are dependent on the phosphorylcholine binding property of CRP.

1). Interactions of CRP with Complement System

The classical complement pathway can be activated by CRP-CPS complexes, aggregated human CRP or by complexes of CRP with phosphorylcholine-containing or cation-containing ligands (Kaplan and Volanakis, 1974; Claus et al., 1977a; Claus et al., 1977b; Richards et al., 1979). The functional and biological effects of complement activation generated by these complexes include opsonization and lysis (Mortensen

and Gewurz, 1976a; Mold et al., 1982). Phosphorylcholine has been shown to inhibit this complement activating activity of CRP (Kaplan and Volanakis, 1974). Therefore, the phosphorylcholine binding domain of CRP has been suggested to be involved in the complement activating process.

It has been demonstrated that human CRP selectively deposits on damaged tissue and necrotic cells, and binds to membranes which have been structurally altered (Volanakis and Wirtz, 1979; Narkates and Volanakis, 1982; Volanakis and Narkates, 1981). The binding of rat CRP to multilamellar phosphatidylcholine liposomes has been shown to require the disruption of the liposome structure by the incorporation of lysophosphatidylcholine (Nagpurkar et al., 1983). The binding is specific towards the polar phosphorylcholine head groups on the surface of the liposomes. Similar results were obtained from the binding studies of human CRP to unilamellar liposomes (Volanakis and Wirtz, 1979). More importantly, subsequent to the binding of CRP to damaged or altered cell membranes, the complement pathway was shown to be activated (Volanakis and Wirtz, 1979). It has therefore been suggested that CRP recognizes damaged cells <u>in situ</u>, and by subsequently activating the complement pathway, generates the chemotactic and opsonic activities required to promote phagocytosis, leading eventually to the resolution and repair of the lesion (Volanakis, 1982).

2). **Interactions of CRP with Lymphoid System**

A possible interaction between CRP and lymphoid cells was first suggested in 1937 by Abernethy and Francis when they reported a delayed skin reaction to CPS in patients with elevated serum levels of CRP (Abernethy and Francis, 1937). This suggestion was supported by the observation of Wood et al (1953). They found that, while immunizing rabbits, animals which produced CRP in response to the injection of antigen had higher titer of specific antibody against the antigen than those which failed to produce CRP. In addition, Williams et al. (1980, 1982) reported the presence of CRP on the surface of 10-35% of peripheral lymphocytes of patients with rheumatic fever, poststreptococcal cholera, or acute streptococcal infections.

A number of *in vitro* experiments have provided more direct evidence of the interaction of CRP with T-lymphocytes. Fluorescein-labeled CRP was shown to bind to peripheral blood lymphocytes (Oishi et al., 1973). The binding of CRP to lymphocytes could be inhibited by phosphorylcholine or by pre-treatment of the cells with phospholipase C (Hornung, 1972). Mortensen et al. (1975) observed that purified human CRP inhibited rosette formation, proliferative responses to soluble antigens and to allogenetic cells in mixed lymphocyte culture. Further experiments demonstrated a selective binding of human CRP to

antigen-stimulated lymphocytes (Croft et al., 1976). The amounts of migration inhibitory factor and macrophage chemotactic factor synthesized by antigen-stimulated and mitogen-stimulated lymphocytes decreased when the lymphocytes were cultured in the presence of CRP (Mortensen et al., 1977).

3). **Effect of CRP on the Activity of Phagocytic Cells**

In 1951, Wood reported that modest concentrations of purified human CRP or acute phase sera with low levels of CRP markedly stimulated the migration of human polymorphonuclear leucocytes, whereas high concentrations of human CRP inhibited this migration (Wood, 1951). Hokama et al. showed that the rate and degree of neutrophil phagocytosis of strains of Streptococci pneumoniae and Serratia Marcescens and of carbonyl iron spherules in whole blood were increased by the addition of human CRP (in the range of between 10 and 30 μg/ml) (Hokama et al., 1962). Subsequently, Ganrot and Kindmark (1969) and Kindmark (1971) demonstrated the phagocytosis-promoting effect of CRP in serum-free in vitro assay systems using a wide range of bacterial species, and succeeded in recovering CRP from bacterial surfaces (Kindmark, 1972). In addition, Mortensen et al. (1976) observed that the number of monocytes ingesting CPS-coated erythrocytes was reduced from 82% to 13% when CRP was removed from the erythrocytes that had reacted with CRP and

complement. Re-addition of CRP restored the ingestion to 76%, indicating a requirement of CRP in triggering phagocytosis.

Recently, rat CRP has been shown to occur as a membrane-associated protein constitutively expressed on liver macrophages (Kupffer cells) (Kempka et al., 1990). It functions as a galactose-specific receptor for the binding of particulate ligands. This membrane bound rat CRP has been shown to be identical to serum rat CRP and could be functionally replaced by purified rat CRP.

4). **Effects of CRP on Platelet Functions**

Gewurz and Fiedel were the first to demonstrate that human CRP inhibits platelet aggregation stimulated by ADP, epinephrine, or thrombin (Fiedel and Gewurz, 1976a; Fiedel and Gewurz, 1976b). Their results suggested that the inhibitory effect of human CRP on platelet activities might be due to an interference with prostaglandin metabolism (Fiedel et al., 1977). It was afterwards shown that a low molecular weight factor in CRP preparations was essential in this inhibitory effect of human CRP (Fiedel et al., 1982a).

Subsequently, rat CRP was shown to inhibit platelet aggregation induced by platelet-activating factor (PAF) in a dose-dependent manner (Nagpurkar et al., 1988). Rat platelets are normally refractory to aggregation by PAF. However, when rabbit antiserum against rat CRP was added to

rat platelet-rich plasma, aggregation of rat platelets by PAF was observed. In addition, rat CRP also inhibited PAF-induced aggregation of human platelets in a dose-dependent manner. It was suggested that the high concentration of rat CRP present in normal rat serum could be the cause of the refractory response of rat platelets to PAF. Like rat CRP, rabbit and human CRP have also been shown to inhibit platelet aggregation induced by PAF (Vigo, 1985; Kilpatrick and Virella, 1985).

Using an HPLC-gel filtration technique, rat CRP has been shown to bind to PAF (Randell et al., 1990a). The formation of the rat CRP-PAF complex was calcium dependent and could be inhibited by phosphorylcholine, suggesting the involvement of the phosphorylcholine binding domain of rat CRP. The binding of CRP to PAF has been used to explain the inhibitory effect of CRP on PAF-induced aggregation of platelets (Randell et al., 1990a).

Rat CRP has been shown to bind to platelets (Randell et al., 1990b). It has been suggested that the binding of CRP to the surface phospholipids of platelets through its phosphorylcholine binding domain may contribute to its inhibition of platelet activities (Fiedel et al., 1976c; Randell et al., 1990b).

5). **Interaction of CRP with Plasma Lipoproteins**

Using immobilized rat CRP on Sepharose 4B, it was shown

that rat CRP selectively bound apo B and E containing lipoproteins (Saxena et al., 1987a). Human and rabbit CRP have been shown to have similar reactivity with apo B containing lipoproteins (de Beer et al., 1982b; Rowe et al., 1984a). This binding of lipoproteins was calcium dependent and the bound lipoproteins were eluted by phosphorylcholine. Therefore, it presumably involves the phosphorylcholine binding domain of CRP.

In rabbits, some or all of the CRP in acute phase serum exists as a complex with either low density lipoproteins (LDL) or very low density lipoproteins (VLDL) (Pontet et al., 1979; Cabana et al., 1982). The formation of these complexes depends on the concentration of apo B containing lipoproteins in the serum (Rowe et al., 1984a). In humans, CRP co-exists in the circulation with plasma lipids or lipoproteins, but does not bind to them or form complexes to any appreciable extent (de Beer et al., 1982b). However, in the serum of patients with type III hyperlipoproteinemia, which contains the abnormal lipoprotein β-VLDL, the formation of CRP-lipoprotein complexes was demonstrated (Rowe et al., 1984b).

Investigations on the effect of rat CRP on the binding of human LDL to LDL receptors on liver cell membranes from estradiol-treated rats showed that rat CRP inhibited the binding of LDL to these receptors (Saxena et al., 1986). It was shown that this inhibitory effect of rat CRP could be

due to the formation of a LDL-rat CRP complex, rather than the binding of rat CRP to the LDL receptors (Saxena et al., 1986).

Immobilized rat CRP has been tested for its effectiveness to bind plasma VLDL and LDL using a extracorporeal plasmapheretic system with rabbits (Saxena et al., 1990). Results showed that Sepharose-rat CRP columns bound some circulating plasma lipoproteins and most (>90%) of the bound lipoprotein fraction contained VLDL and LDL.

The interaction between CRP- and apo B-containing lipoproteins may have important in vivo functional effects. By binding to damaged cell membranes and then selectively interacting with apo B-containing lipoproteins, CRP may contribute to the specific localization of lipoprotein particles required for active cellular metabolism or processes of repair (Pepys et al., 1985). This may be relevant to the pathogenesis of atherosclerosis.

6). **CRP in Defence Mechanisms against Infection and the Clearance of Abnormal Materials from Circulation**

The phosphorylcholine binding property of CRP enables it to recognize and bind a wide range of either microorganisms or their degradation products. It has been suggested that CRP may reduce the toxicity of the microorganisms by simply binding to them. Besides, secondary processes such as complement activation and phagocytosis may

also contribute to protection against infection (Yother et al., 1982). This role of CRP has been demonstrated by an experiment in which human CRP was shown to protect mice from lethal Streptococcus pneumoniae infection (Mold et al., 1981a). This result supports the concept that CRP may act in this way both in certain primitive animals, which have not evolved specific antibody mechanisms, and in more developed animals at an early stage of infection, before the production of specific antibodies get under way.

Studies on the interaction between CRP and certain abnormal materials have also provided evidence that support the role of CRP in defense mechanisms (Pepys, 1981). Human CRP has been shown to bind to chromatin in the presence of calcium (Robey et al., 1984). It has also been shown that human CRP mediates the solubilization of chromatin by complement (Robey et al., 1985). In addition, CRP from rat and horseshoe crab have been found to bind mercury both in vivo and in vitro, and it has been suggested that CRP may act as a scavenger for toxic metals (Agrawal and Bhattacharya, 1989).

7). Clinical Applications of CRP

During severe infections, CRP concentration in serum increases, and the actual degree of increase usually corresponds with the severity of the infection (Sabel and Hanson, 1974; Moodley, 1981). Based on these observations,

measurement of serum CRP has been used in clinical diagnosis, especially in distinguishing bacterial infections from viral ones, and in the management of a wide range of clinical conditions (Table 2).

Table 2. **Clinical Applications of the Measurement of Serum CRP**

Screening for organic disease
Monitoring the extent and activity of disease
Infection
Inflammation
Malignancy
Necrosis
Detection of infections

(Taken from Pepys and Baltz (1983)).

Section II. GLYCOPROTEIN: AN OUTLINE

Glycoproteins are proteins to which oligosaccharides are covalently attached. It is well known that the carbohydrate chains on glycoproteins play important roles in their biological functions and metabolism. The clearance of glycosylated CRP has been examined in this study. A brief outline on the structural features and carbohydrate-dependent clearance mechanism of glycoproteins is therefore provided in this section.

A. STRUCTURE OF GLYCOPROTEINS

1). Composition of the Carbohydrate Moiety of Glycoproteins

Although at least 200 different monosaccharides have been discovered in nature, only 11 are known to occur in glycoproteins (Sharon and Lis, 1981). Most of these monosaccharides are hexoses or their simple derivatives, such as N-acetylhexosamines and uronic acids. In addition, many animal glycoproteins contain more complex monosaccharides, e.g., the sialic acids. In glycoproteins, these monosaccharides exist as residues in oligosaccharide chains. The reducing groups at the end of these oligosaccharides are involved in the carbohydrate-peptide linkage.

2). Carbohydrate-Polypeptide Linkages

The carbohydrate chains on glycoproteins are classified

according to their linkage to the polypeptide chains (Kornfeld and Kornfeld, 1980). A carbohydrate chain is termed an N-linked or asparagine-linked oligosaccharide if it is attached to the amide group of an asparagine side chain (Fig. 1). On the other hand, if a carbohydrate chain is attached to a hydroxyl group in the side chain of serine, threonine, hydroxylysine or hydroxyproline, it is called an O-linked oligosaccharide (Fig. 1).

In most glycoproteins that have been examined so far, the carbohydrate chains seem to be preferentially attached to peptide sequences forming β-turns (Rademacher et al., 1988). Where N-linked oligosaccharides are attached, the amino acid sequence next to the glycosylated asparagine is always asparaginyl-X-serine/threonine (X can be any amino acid except glycine). In glycoproteins where more than one N-linked oligosaccharide is present, several amino acids separate the sites of attachment of the carbohydrate chains. In contrast, O-linked oligosaccharides may be attached to neighbouring amino acid residues.

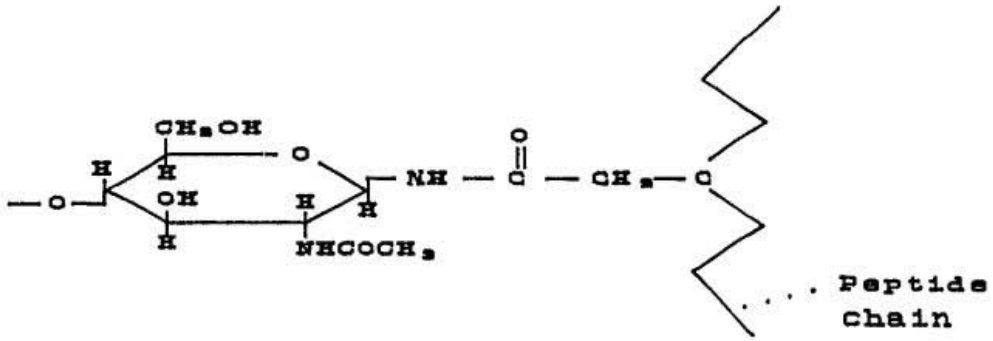
3). Oligosaccharides in Glycoproteins

N-linked oligosaccharides

The N-linked oligosaccharides are by far the most common ones found in glycoproteins (Kornfeld and Kornfeld, 1985). A common feature of N-linked oligosaccharides is that they have a common inner core, consisting of a branched

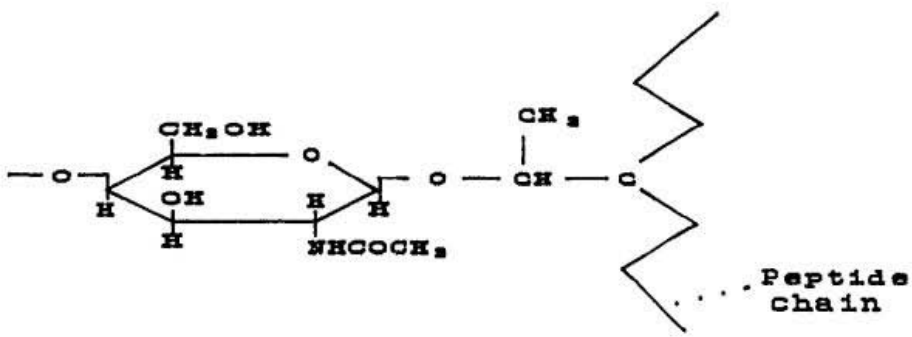
Fig. 1. Major Types of Carbohydrate-peptide Linkage

1. N-glycosidic linkage



GlcNAc ——— Asparagine

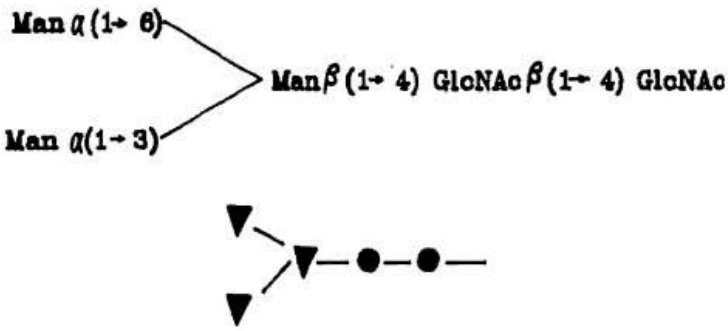
2. O-glycosidic linkage



GalNAc ——— Thr/Ser

pentasaccharide: trimannosyl-di-N-acetylglucosamine [Man3(GlcNAc)2] (Fig. 2).

Fig. 2. The common core of N-linked oligosaccharides



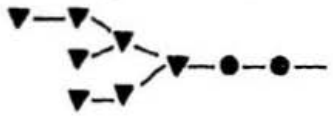
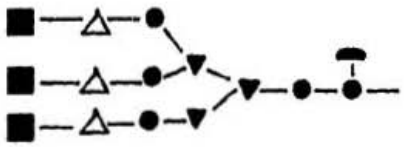
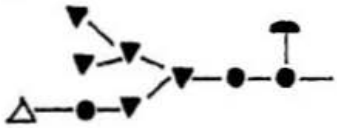
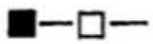
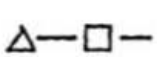
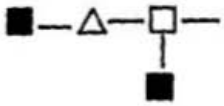
Key: ▼ = Man, ● = GlcNAc

adapted from Sharon and Lis (1981)

The peripheral mannose residues are usually substituted. Due to these substitutions, three major types of N-linked oligosaccharides are formed: the high-mannose type (oligomannosidic type), the complex type, and the hybrid type of oligosaccharides (Kornfeld and Kornfeld, 1985). Typical examples of the three types of N-linked oligosaccharides are shown in Fig. 3.

The high-mannose oligosaccharides typically have two to six additional mannose residues linked to the pentasaccharide core, whereas the complex oligosaccharides contain a diverse group of additional sugars to the core structure. The hybrid oligosaccharides are so named because they have features of both high-mannose and complex

Fig. 3. Typical Examples of Oligosaccharides in Glycoprotein

Type	Examples
N–Glycosidic	
High–mannose oligosaccharides	 Chicken ovalbumin, Sindbis virus
Complex oligosaccharides	 Vesicular stomatis virus
Hybrid oligosaccharides	
O–Glycosidic	
	 Ovine submaxillary mucin
	 Antifreeze glycoprotein, rat and rabbit brain glycoproteins
	 Calf fetuin, Glycophorin

Key: ▼= Man, ●= GlcNAc, △ = Gal, □ = GalNAc, ■ = NeuNAc, ◓ = L–Fuc.

adapted from Sharon and Lis (1981)

oligosaccharides.

The common pentasaccharide of N-linked oligosaccharides may be modified both by the addition of extra sugar residues that elongate the outer chains and by the addition of extra branches to the α mannose residues of the core structure. The outer branches of the oligosaccharides are also often referred to as antennae of the oligosaccharides. The majority of N-linked oligosaccharides contain two, three, or four antennae (Paz-Parente et al., 1982; Yamashita et al., 1983; Francois-Gerard et al., 1983).

The high-mannose oligosaccharides occur in animals, higher plants, yeast, and fungus glycoproteins. On the other hand, complex oligosaccharides appear to be confined to animals. In mammals, no more than six mannose residues attached to the core structure have been found. But more primitive organisms, such as yeast, may contain much larger high-mannose oligosaccharides (Schachter, 1984).

O-linked oligosaccharides

In contrast to N-linked oligosaccharides, few generalizations can be made about O-linked oligosaccharides. The carbohydrate structures vary from a single galactose residue (e.g., in collagen) to branched oligosaccharides of up to 16 to 18 monosaccharide residues (e.g. in soluble blood group substances). Perhaps the only common feature in the structure of O-linked oligosaccharides is the GalNAc

attached to the hydroxyl group of serine or threonine in many O-linked oligosaccharides (Fig. 3).

B. BIOSYNTHESIS OF GLYCOPROTEINS

The polypeptide chains of glycoproteins are synthesized in the same way as non-glycosylated proteins. They are assembled on membrane-bound ribosomes on rough endoplasmic reticulum, under direct control of the genetic code. The initial glycosylation occurs either while the polypeptide is still nascent on the ribosome or shortly after translation has been completed.

Unlike the polypeptide chains, carbohydrate chains in glycoproteins are not primary gene products. They are synthesized by enzymes known as glycosyl transferases (Rademacher et al., 1988). These transferases are bound to the membranes of the endoplasmic reticulum or the Golgi apparatus. They act in a very specific order, without using a template, but rather by recognizing the actual structure of the growing oligosaccharide as the appropriate substrate. This type of synthesis is not very accurate and therefore results in microheterogeneity in the carbohydrate moiety of glycoproteins. For example, α1-acid glycoprotein (AGP) from human serum has at least 19 variant carbohydrate structures at the five attachment sites along the polypeptide chain.

For O-linked oligosaccharides, the sugars are added one at a time to the polypeptide chains. Usually GalNAc is added

first, which is elongated by a variable number of additional sugar residues, ranging from just a few to 10 or more (Ruoslahti, 1988).

Unlike the synthesis of O-linked oligosaccharides, the initial step in the synthesis of N-linked oligosaccharides cannot be accomplished by the addition of individual sugars to the polypeptide chains. Rather, it requires the transfer en bloc of a single species of oligosaccharide, containing 3 Glc, 9 Man and 2 GlcNAc residues, from dolichol pyrophosphate oligosaccharide to an asparagine residue in the polypeptide chains (Kornfeld and Kornfeld, 1985). The oligosaccharides are almost always transferred to growing polypeptide chains, ensuring maximum access to the target asparagine residues.

Once initial glycosylation has occurred within the rough endoplasmic reticulum, the oligosaccharide is trimmed immediately. The trimming process continues as the glycoprotein is transported to Golgi apparatus. The diversity of the oligosaccharide structures on mature glycoproteins results mainly from this extensive modification of the original oligosaccharide (Berger et al., 1982; Kornfeld, 1982; Schachter et al., 1983).

C. CLEARANCE OF GLYCOPROTEINS

Proteins are degraded in a controlled manner during normal growth and differentiation (Rechsteiner et al. 1987).

In addition to features such as conformation of the polypeptide, the carbohydrate moiety has been shown to play an important role in regulating the catabolism of glycoproteins. Several carbohydrate-dependent clearance pathways for glycoproteins have been discovered.

1). **Clearance of Glycoproteins through the Hepatic Asialoglycoprotein Receptor**

In the late 60's, Ashwell and Morell discovered that the clearance rate of ceruloplasmin, a copper transporting glycoprotein in serum, was dramatically enhanced when its terminal sialic acids were removed by neuraminidase treatment (Ashwell and Harford, 1982). The asialo-ceruloplasmin was rapidly taken up and degraded in the liver. Similar results were obtained with other mammalian serum glycoproteins. Detailed investigations of this phenomenon revealed a receptor for asialoglycoproteins on the surface of hepatocytes (the hepatic asialoglycoprotein receptor). This receptor recognizes the terminal galactose residues of a variety of asialoglycoproteins and mediates the uptake of asialoglycoproteins into lysosomes for hydrolysis. Thus, a clearance pathway for mammalian circulating glycoproteins has been established, which is often referred to as Ashwell's pathway (Fig. 4).

The hepatic asialoglycoprotein receptor has been isolated and purified (Schwartz and Ashwell, 1984). Due to

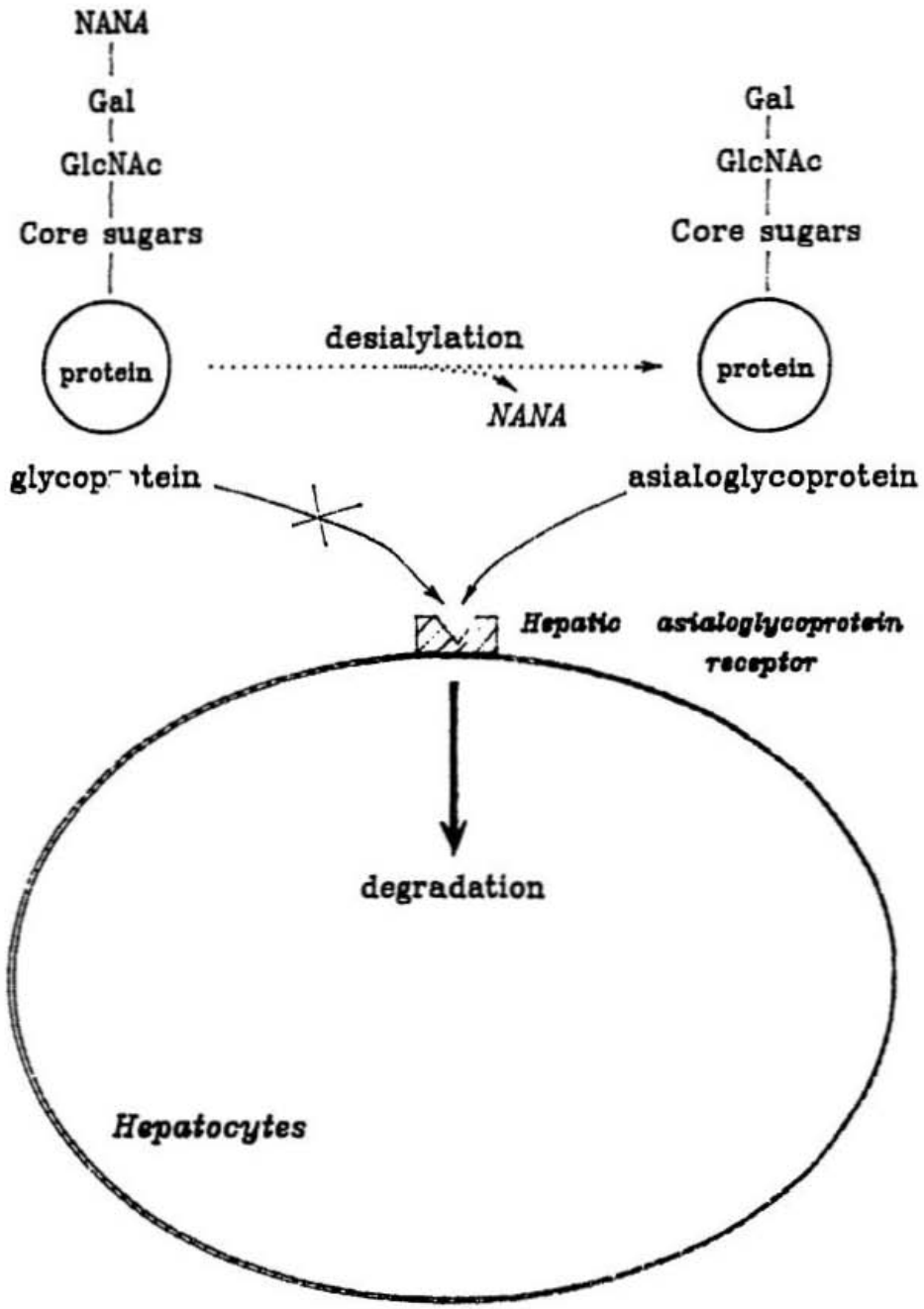


Fig. 4. Ashwell's pathway

its lectin-like specific binding to galactose and the fact that the binding can be specifically inhibited by GalNAc, this receptor is also called hepatic Gal/GalNAc specific lectin. It is the first animal lectin discovered and characterized. Similar to plant lectins, this lectin also agglutinates neuraminidase-treated cells and is mitogenic for lymphocytes. These properties imply multi-valency of binding sites on one functional lectin molecule.

The binding and uptake of asialoglycoproteins into hepatocytes also seem to rely on multiple interactions of the asialoglycoproteins with the hepatic asialoglycoprotein receptor (Baenziger and Fiete, 1980). Asialoglycoproteins or purified asialoglycopeptides with multiple terminal galactose residues are bound with high affinity to this receptor and are rapidly taken up into the cell. Asialoglycoproteins with two or less terminal galactose residues usually bind poorly. Thus asialo AGP, which has many tri- or tetraantennary N-linked oligosaccharides, is an excellent substrate for this receptor, whereas serum asialotransferrin with biantennary oligosaccharides is a poor substrate (Hatton et al., 1979).

Sialic acids have been shown to play a dual role in this clearance pathway (Hudgin et al., 1974). On one hand, the removal of terminal sialic acids from glycoproteins is a pre-requirement for their binding to the hepatic asialo-

glycoprotein receptor. On the other hand, the presence of sialic acids on the receptor is necessary to keep the receptor biologically functional. Neuraminidase treatment of the hepatocytes or the purified hepatic asialoglycoprotein receptor resulted in complete inhibition of the interaction between asialoglycoproteins and the hepatic receptor. The removal of sialic acids from the carbohydrate chains of the hepatic receptor induces self-aggregation through interaction with exposed galactose terminal residues, and impairs its binding of asialoglycoproteins.

2). **Other Carbohydrate-dependent Clearance Mechanisms**

Clearance systems in which sugars other than galactose serve as determinants have been identified. Besides hepatocytes, non-parenchymal cells in the liver, particularly Kupffer cells, also play an important role in the clearance of glycoproteins.

After AGP has been successively treated with neuraminidase and β-galactosidase to expose its GlcNAc residues, the modified protein is rapidly taken up by the reticuloendothelial system in the liver. Mannose-terminated glycoproteins are cleared in a similar way (Stahl et al., 1976). Therefore, the lectin on the surface of Kupffer cells binds glycoproteins carrying either terminal GlcNAc or Man residues.

An analogous lectin is also widely distributed on phagocytic cells, e.g. alveolar macrophages. The function of the above mentioned lectins may include the clearance of aged or partially degraded serum glycoproteins from the circulation, and the destruction of microorganisms, such as yeasts, which are rich in oligomannosidic chains. The ingestion of yeasts by alveolar macrophages, for example, is inhibited by mannose and glycoproteins with terminal mannose residues (Warr, 1980).

Unlike mammalian serum glycoproteins, normal circulating glycoproteins in avian species usually possess exposed galactose residues. Obviously, therefore, clearance via the hepatic Gal/GalNAc specific lectin cannot exist in these species. It was found that in these species a clearance system which recognizes terminal GlcNAc residues of glycoproteins operates on the surface of the hepatocytes (Regoeczi et al., 1975). The elegant work of Regoeczi and his co-workers showed that the half-life of avian glycoproteins was dramatically decreased after being treated with β-galactosidase to expose their penultimate GlcNAc residues (Regoeczi et al., 1975).

It has been found that the carbohydrate binding domain of several cloned animal lectins share some common features in their amino acid sequences (Drickamer, 1988). Homologous carbohydrate binding domains have also been found in other

animal lectins. By searching the known protein sequences for the carbohydrate binding motif, additional animal lectins have been discovered, including pulmonary surfactant protein A, proteoglycan core proteins, and lymphocyte receptor for the Fc portion of IgE (Drickamer, 1988).

Section III. **PURPOSE OF THE PRESENT STUDY**

As described earlier in this chapter, CRP investigated so far are all synthesized mainly in the liver. However, their catabolic pathway is not clear. Based on Ashwell's discovery, the hepatic asialoglycoprotein receptor is a potential site for the clearance of mammalian circulatory glycoproteins. Rat CRP, being a serum glycoprotein, offered us an opportunity to study its clearance by Ashwell's pathway.

Since not all CRP are glycosylated, it is appropriate to determine if glycosylated CRP and non-glycosylated CRP follow different clearance pathways. CRP binds to phosphorylcholine, and the cell surface is abundant with phosphorylcholine residues. It is therefore relevant to examine if the phosphorylcholine binding property of CRP would affect the binding of asialo CRP to the hepatic asialoglycoprotein receptor and the subsequent clearance of these proteins through this receptor.

In the present study, the binding of rat asialo CRP, rat CRP and rabbit CRP to isolated hepatocytes has been examined. The clearance of these proteins has been investigated *in vivo* and also using perfused rat liver. Competitive inhibition experiments have been carried out *in vitro* and *in vivo* between rat asialo CRP and asialo AGP. The

effect of GalNAc and phosphorylcholine on the binding of rat asialo CRP has been studied, rat and rabbit CRP to isolated hepatocytes and on the clearance of these proteins by perfused liver.

CHAPTER 2 MATERIALS AND METHODS

A. MATERIALS

1). **Animals** Male Sprague-Dawley rats (body weight 250-300 g) and 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 chow ad libitum.

2). **Reagents and Chemicals** Waymouth's MB 752/1 medium (1X) was from Gibco laboratories, USA. $Na^{125}I$ (reductant free, 2mCi in 4-5 μl 0.1 M NaOH) solution was from Du Pont Canada Inc. Cyanogen bromide (CNBr) activated Sepharose 4B was from Pharmacia. Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) was from Pierce. Neuraminidase from Cl. perfringens (EC 3.2.1.18), Collagenase from Cl. histolyticum, type IV (EC 3.4.24.3), α_1-acid glycoprotein (Bovine), Sephadex G-25-150 (bead size 50-150 μ) and all other chemicals were from Sigma Chemical Company.

3). **Medium and Buffer** Medium/BSA: Waymouth's MB 752/1 medium plus 0.2% (w/v) bovine serum albumin (BSA) (pH 7.4). Hanks' buffer: 0.137 M NaCl, 26 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.5 mM EGTA, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.4 mM KCl (pH 7.4). Kreb's-bicarbonate/BSA: 118 mM NaCl, 23.8 mM $NaHCO_3$, 0.95 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 4.8 mM KCl, 2.9 mM

$CaCl_2 \cdot 2H_2O$, 0.25% BSA (pH 7.4). Tris buffer: 5 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and 2 mM Ca^{2+}.

B. **PREPARATIVE PROCEDURES**

1). **Preparation of Sepharose-phenylphosphorylcholine Affinity Adsorbent** The affinity adsorbent for the isolation and purification of rat and rabbit CRP was prepared as described by Nagpurkar and Mookerjea (1981). Typically, 4-nitrophenyl-phosphorylcholine (0.6 g, 1.19 nmol) in methanol (50 ml) was reduced with H_2 at 1 atm using 0.2 g of 5% palladium on charcoal as catalyst for 2 h at room temperature. The reaction mixture was filtered and the filtrate evaporated under reduced pressure. The resulting 4-aminophenylphosphorylcholine was immediately dissolved in 100 ml of 0.1 N $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and was added to 15 g of CNBr-activated Sepharose 4B which had been previously washed with 1 mM HCl. The mixture was gently mixed for 4 h at room temperature, filtered and then washed alternatively with 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and 0.1 M CH_3COONa buffer (pH 4.2) containing 0.5 M NaCl. The Sepharose-phenylphosphorylcholine was treated overnight at 4°C with 200 ml of 1 M ethanolamine (pH 9.0) and finally re-suspended in 5 mM Tris-HCl (pH 7.8) and stored at 0-4°C.

2). **Preparation of Rat and Rabbit Sera** Rats were anaesthetized lightly with ether, the abdomen cut open and blood obtained from the abdominal aorta. The blood was allowed to clot for 1 hour at room temperature followed by another hour at 4°C. It was then centrifuged for 5 min at 1,000 xg in a bench top centrifuge. The serum was taken out and used immediately or stored at -20°C until use.

Inflammation was induced in rabbits by subcutaneously injecting turpentine (0.5 ml/kg body weight) in the thighs. Blood was obtained after 48 h by cardiac puncture from rabbits under anaesthesia induced by intraperitoneal injection of sodium phenobarbital (1 ml/kg body weight). Serum was prepared as described for rats.

3). **Isolation and Purification of Rat and Rabbit CRP** Rat CRP and rabbit CRP were isolated and purified from normal rat serum and inflamed rabbit serum, respectively, according to the procedure of Nagpurkar and Mookerjea (1981). Sepharose-phenylphosphorylcholine affinity adsorbent was packed in a column (20 x 2 cm) and equilibrated with 5-10 bed volume of Tris buffer at a flow rate of 1 ml/min. Serum (<40 ml) was brought to a concentration of 5 mM with respect to Tris-HCl, 2 mM with respect to Ca^{2+}, and applied on the column. The column was washed extensively with Tris buffer until absorbance at 280 nm of the eluent was less than 0.02. CRP was then eluted with 8 mM phosphorylcholine in Tris

buffer. After dialysing exhaustively against Tris buffer (4 changes), the protein solution was concentrated by ultra-filtration, and aliquots were stored at -20°C. Protein concentration was measured by the method of Lowry et al. (1951) using BSA as standard. The purity of CRP was checked by electrophoresis and high performance liquid chromatography (HPLC). Preparations which gave a single band on electrophoresis or a single peak on HPLC were used for further studies.

4). **Preparation of Asialo Proteins** Rat CRP (10 mg) was desialylated by incubating with 0.5 international activity units of neuraminidase in 10 ml of Tris buffer at 37°C for 3 h. The incubation mixture was then applied to a Sepharose-phenylphosphorylcholine affinity column (2 cm x 35 cm). The column was washed extensively with Tris buffer. Rat asialo CRP bound to the column was eluted with 8 mM phosphoryl-choline in Tris buffer. AGP and fetuin were desialylated by treating with 0.05 M H_2SO_4 at 80°C for 60 min, following the procedure of Spiro (1964). The amount of sialic acid released during incubation and sialic acid remaining on the protein was determined using the method of Warren (1959). Typically, desialylation resulted in the removal of >90% of the sialic acid from rat CRP and AGP.

5). **Radioiodination Procedures** Rat CRP, rat asialo CRP, rabbit CRP, AGP, and asialo AGP were iodinated with Iodo-Gen

iodination reagent as the catalyst following the manufacturer's procedure (Pierce). Briefly, 2 mCi of carrier free $Na^{125}I$ was diluted to 60 µl with 0.1 N NaOH. Iodo-Gen coated tubes were rinsed with Tris buffer. Protein solution in Tris buffer was then added, to a ratio of 10 µg protein/µg Iodo-Gen, followed by 500 µCi $Na^{125}I$ in 15 µl of 0.1 N NaOH. The reaction mixture was left at room temperature for 10-15 min with occasional shaking, and then applied to a G-25 column (20 ml bed volume), which had been previously equilibrated with Tris buffer followed by 0.5 ml of BSA (1 mg/ml in Tris buffer). The labeled protein was collected from the void volume and dialysed against Tris buffer. Samples were aliquoted and stored at -20°C. The specific radioactivity was in the range of 1.0-1.5 x 10^6 CPM/µg protein and greater than 96% of the radioactivity was precipitable by ice-cold trichloroacetic acid (5%)-phosphotungstic acid (1%).

C. **ANALYTICAL PROCEDURES**

1). **Electrophoresis** Protein samples were analyzed by either native or SDS polyacrylamide gel electrophoresis (Native-PAGE, and SDS-PAGE) using the separation unit of a PhastSystem (Pharmacia) and 8-25% gradient gels (PhastGel, Pharmacia). After electrophoresis, the gels were stained with Coomassie R 350 dye (Pharmacia) using the development unit of the PhastSystem.

To determine whether the iodination procedure had altered the proteins, labeled protein sample was mixed with an equal volume of unlabeled protein. The electrophoresis was run under the same condition as with unlabeled proteins. After staining with Coomassie blue R 350 dye, the gel was manually cut into 2-3 mm sections and counted for radioactivity in a gamma counter. Over 90% of the radioactivity appeared in the region of the unlabeled proteins.

2). **HPLC Analysis** HPLC was performed on a Perkin-Elmer Series 4 HPLC system using a TSK-250 gel filtration column (300 x 7.5 mm, Bio-Rad). The column was equilibrated with 5-10 bed volumes of 0.05 M Na_2SO_4-0.02 M NaH_2PO_4 buffer (pH 6.8) at a flow rate of 1.0 ml/min. A protein sample (containing 10-100 μg protein in 10-100 μl) was applied and the column eluted at a flow rate of 1.0 ml/min with the equilibration buffer. The eluent was monitored at 280 nm.

The ^{125}I-labeled proteins were analysed by HPLC to determine wether iodination procedure had altered the protein. Labeled protein sample was mixed with an equal volume of unlabeled protein. The mixture was applied to the column and eluted as described above. The eluent was monitored for its absorbance at 280 nm and for radioactivity with a Beckman 171 radioisotope detector. Alternatively, fractions (0.5 ml) of the eluent were collected and counted for radioactivity in a Beckman gamma 5500 counter. More than

90% of the radioactivity was eluted under the peak for the unlabeled protein.

The following procedure was used to analyze the radioactivity associated with tissue homogenates (see part D "in vivo experiments" for the preparation of tissue homogenates) after the injection of radioactive protein samples. Aliquots of 1 ml of liver and lung homogenates were transferred to Eppendorf tubes. To release the bound radioactivity from the tissue, EDTA solution (0.12 ml, 100 mM) was added. The total volume was adjusted to 1.5 ml with saline. After incubating at room temperature for 15 min, the samples were centrifuged at 11,000 rpm for 5 min. The supernatant was filtered through a 0.22 μm filter and applied on an HPLC gel filtration column (Spherogel-TSK, 7.5 x 300 mm), which had been previously equilibrated with Na_2SO_4/NaH_2PO_4 buffer (pH 6.8). The flow rate was maintained at 1.0 ml/min. The eluent was monitored at 280 nm and fractions of the eluent were collected and counted for radioactivity in a gamma counter.

D. IN VIVO CLEARANCE EXPERIMENTS

Rats were anaesthetized with ether and a protein sample (10-15 μg, 8-15 x 10^6 CPM in 250 μl Tris buffer) was injected through a tail vein. The tip of the tail was cut and blood samples were collected at regular time intervals into tubes containing 50 μl of 10 μg/μl heparin in saline. The volume of each blood sample collected was measured.

Duplicate aliquots of the blood samples (30 µl) were taken into counting vials containing 1.0 ml saline and counted for radioactivity in a gamma counter. After the last blood sample was taken, the remaining blood in the animal was removed by perfusing through the vena cava with Hanks' buffer. Liver, lungs, kidney, spleen, pancreas, thymus, heart, stomach, and large and small intestine were removed, rinsed with ice-cold saline, blotted dry, weighed, and homogenised in ice-cold saline with a Polytron homogenizer (setting 6, for 10 sec, x 3 times). The total volume of each homogenate was measured. Duplicate samples of 0.8 ml tissue homogenates were transferred to Eppendorf tubes containing 0.6 ml saline. After centrifuging at 11,000 rpm for 3 min, the radioactivity in the supernatant and pellet of each sample was measured in a gamma counter. The radioactivity associated with the pellet was taken as tissue associated radioactivity. The radioactivity in each whole organ and for per gram tissue was then calculated.

To determine the protein-bound radioactivity, 0.4 ml of tissue homogenate or 30 µl of blood sample was incubated for 15 min at 0-4°C with 1 ml of ice-cold trichloroacetic acid (5%)-phosphotungstic acid (1%). After centrifuging, the radioactivity associated with the pellets was counted for radioactivity in a gamma counter. The radioactivity precipitable by ice-cold trichloroacetic acid (5%)-phosphotungstic acid (1%) was taken as protein-bound

radioactivity.

E. LIVER PERFUSION EXPERIMENTS

Rats were anaesthetized with ether and the abdominal cavity cut open. The liver was then perfused through the portal vein with Hanks' buffer at 37°C, during which time the liver was excised free of other tissue and placed in a reservoir. The perfusion solution was then changed to 70 ml of Waymouth's medium containing the labeled protein sample and recirculated at 37°C for 45-60 min through the liver at a flow rate of 30 ml/min. During the perfusion, the solution was constantly oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide. Samples of the perfusate (0.2 ml) were taken at regular time intervals. At the end of the experiment, the perfusion solution was changed to saline and the liver was briefly perfused to remove the remaining Waymouth's medium. The radioactivity in 50 µl aliquots of the perfusate samples and in the liver was determined as described earlier.

Effect of GalNAc or phosphorylcholine on the clearance of labeled rat asialo CRP and asialo AGP was studied by including 8 mM GalNAc or 1 mM phosphorylcholine in Waymouth's medium. When studying the effect of neuraminidase on the hepatic asialoglycoprotein receptor, the liver was first perfused at 37°C with 70 ml of Waymouth's medium containing 0.5 international activity units of neuraminidase

for 30 min. The liver was then perfused with 30 ml of Waymouth's medium to remove the remaining neuraminidase. The perfusion solution was subsequently changed to 70 ml of fresh Waymouth's medium containing 15 μg of labeled rat asialo CRP or asialo AGP, and the liver perfused for another 45 to 60 min. The radioactivity in the perfusate was monitored as described earlier.

F. PREPARATION OF HEPATOCYTES

Rat hepatocytes were prepared by a modification of collagenase perfusion procedure of Seglen (1973). Male Sprague-Dawley rats (200-250 g body weight) were anaesthetized with ether and the abdominal cavity exposed. The liver was then perfused through the portal vein with calcium-free Hanks' buffer at 37°C, during which time the liver was excised free of other tissue and placed in a reservoir. The perfusion solution was changed to 120 ml of Hanks' buffer, containing 25 mg of collagenase and 35 mg of $CaCl_2 \cdot 2H_2O$ and re-circulated through the liver at a flow rate of 30 ml/min for about 10-15 min at 37°C. The perfusion solution was constantly oxygenated with a gas mixture of 95% oxygen and 5% carbon dioxide. After perfusing with collagenase solution, the liver was placed in a 100 mm Petri dish on ice, rinsed with ice-cold Medium/BSA, then squeezed gently by hand to release the cells. The cell suspension was incubated at 37°C for 30 min with 95% oxygen and 5% carbon

dioxide as the gas phase. Subsequently, the cell suspension was kept on ice for 10 min to bring the temperature down to 0-4°C, and then filtered through 8 layers of cheese-cloth to remove cell aggregates. The filtrate was washed three times by centrifuging at 50 xg for 2 min. The final cell pellet was re-suspended in ice-cold Medium/BSA and the cell concentration was determined using a Levy chamber. Cell viability, assessed by 0.02% trypan blue exclusion, was usually 80-85%. Hepatocytes with different viability were prepared by leaving the cells in a hypotonic buffer (26 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 5.4 mM KCl, pH 7.4) for different periods of time.

G. HEPATOCYTE BINDING ASSAYS

Binding assays were carried out following the procedure described by Weigel (1980) but with certain modifications. In order to prevent internalization of the surface bound proteins, a temperature of 4°C was chosen in the binding assays. The binding at this temperature represents surface binding (Tolleshaug, 1981). In a typical binding assay, hepatocytes (6.5×10^5) and labeled protein sample were incubated in 1 ml of Medium/BSA at 4°C for 90 min. The tubes used were pre-treated with 0.5% BSA for 1 h to prevent non-specific binding to the wall of the tubes. After adding the cell suspension, the tubes were gassed with 95% oxygen and 5% carbon dioxide mixture, and immediately stoppered. Cells

were kept in suspension by gently swirling the tubes about every 5 min. At the end of the incubation, the incubation mixtures were first diluted with about eight volumes of ice-cold Kreb's-bicarbonate/BSA buffer, then centrifuged at 2,000 rpm for 2 min. After removing he supernatant by aspiration, the pellet was gently suspended in 10 ml of ice-cold Kreb's-bicarbonate/BSA and re-centrifuged. This procedure was repeated twice. The final pellet was transferred into a Bio-Vial counting vial, and counted for radioactivity in a gamma counter. The radioactivity associated with the pellet was taken as the total binding. Non-specific binding was determined by carrying out the binding assays in the presence of a 100-fold excess of the unlabeled protein or in the presence of 1 mM phosphoryl-choline. For each concentration of the labeled protein, the specific binding was obtained by subtracting the non-specific binding from the corresponding total binding. The optimum cell concentration and incubation time at 37°C and 4°C was established by carrying out binding assays with increasing cell concentration or different incubation times. The optimum cell concentration chosen was on the linear portion of binding curve, while the optimum incubation time selected produced maximum binding. The results of the binding assays were reproducible and are representative of at least two separate experiments.

Chapter 3 RESULTS

Section I. BINDING OF RAT ASIALO CRP, RAT CRP AND RABBIT CRP TO ISOLATED HEPATOCYTES

Isolated hepatocytes have previously been used in studies on the binding of asialoglycoproteins to the hepatic asialoglycoprotein receptor (Morell et al., 1971; Ashwell and Harford, 1982; Weigel, 1980). To examine the binding of glycosylated CRP to this receptor, the binding of rat CRP and rat asialo CRP to isolated hepatocytes was studied. Since most of the studies on the asialoglycoprotein receptor are based on the binding of asialo AGP to isolated hepatocytes, this protein was therefore used as a positive control in this study. Competitive inhibition studies of the binding of rat asialo CRP and asialo AGP to the hepatocytes were carried out in order to determine if these proteins bind to the same receptor on the hepatocytes. In addition, the effect of phosphorylcholine and GalNAc on the binding of rat asialo CRP and asialo AGP to the hepatocytes was examined.

A. PURITY AND IDENTITY OF RAT CRP, RAT ASIALO CRP AND RABBIT CRP

The purity of isolated rat CRP and rabbit CRP was analyzed by HPLC using a gel-filtration column. Isolated rat CRP gave a single major peak which accounted for more than

95% of the total peak area (Fig. 5). A similar HPLC profile was obtained with isolated rabbit CRP.

After neuraminidase treatment, 93% of the sialic acid on rat CRP was removed. The resulting rat asialo CRP cross-reacted with rabbit antiserum against rat CRP. It gave a single peak on HPLC, and retained the phosphorylcholine binding property, as was confirmed by its binding to Sepharose-phenylphosphorylcholine column (Fig. 6).

B. CHARACTERIZATION OF ^{125}I-LABELED PROTEINS

The ^{125}I-labeled proteins were analyzed on an HPLC system with a Beckman 171 radioisotope detector. Fig. 7 shows that the elution position of the ^{125}I-labeled rat CRP corresponds to that of unlabeled rat CRP monitored at 280 nm. Similar results were obtained for ^{125}I-labeled rat asialo CRP, rabbit CRP, and asialo AGP. The purity of the ^{125}I-labeled proteins was also checked by electrophoresis on native 8-25% gradient polyacrylamide gels. In each case, the ^{125}I-labeled proteins had the same mobility as unlabeled proteins.

The labeled CRP bound to Sepharose-phenylphosphoryl-choline column and could be eluted with 8 mM phosphoryl-choline solution (Fig. 8). Fractions of the elutent was counted for radioactivity with a Beckman gamma 5500 counter. Greater than 95% of the radioactivity corresponded to the

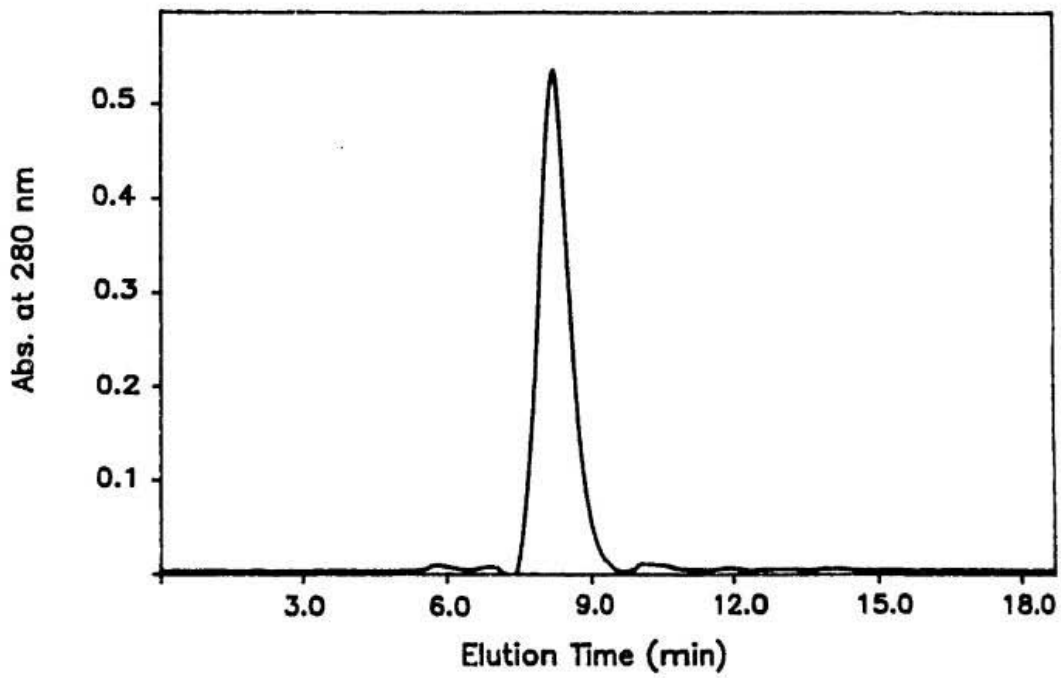


Fig. 5. **HPLC profile of isolated rat CRP.** A sample of isolated rat CRP in Tris buffer (100 µl, 0.89 mg/ml) was applied to a HPLC gel filtration column (300 x 7.5 mm, Bio-Rad) which had been equilibrated with 10 bed volumes of 0.05 M Na_2SO_4-0.02 M NaH_2PO_4 buffer (pH 6.8) at a flow rate of 1.0 ml/min on a HPLC system (Perkin-Elmer Series 4). The column was washed at a flow rate of 1.0 ml/min with the equilibration buffer and the eluent monitored at 280 nm. This graph has been regenerated by digitizing the elution profile with Sigmaplot program 3.1 (Jandel Scientific).

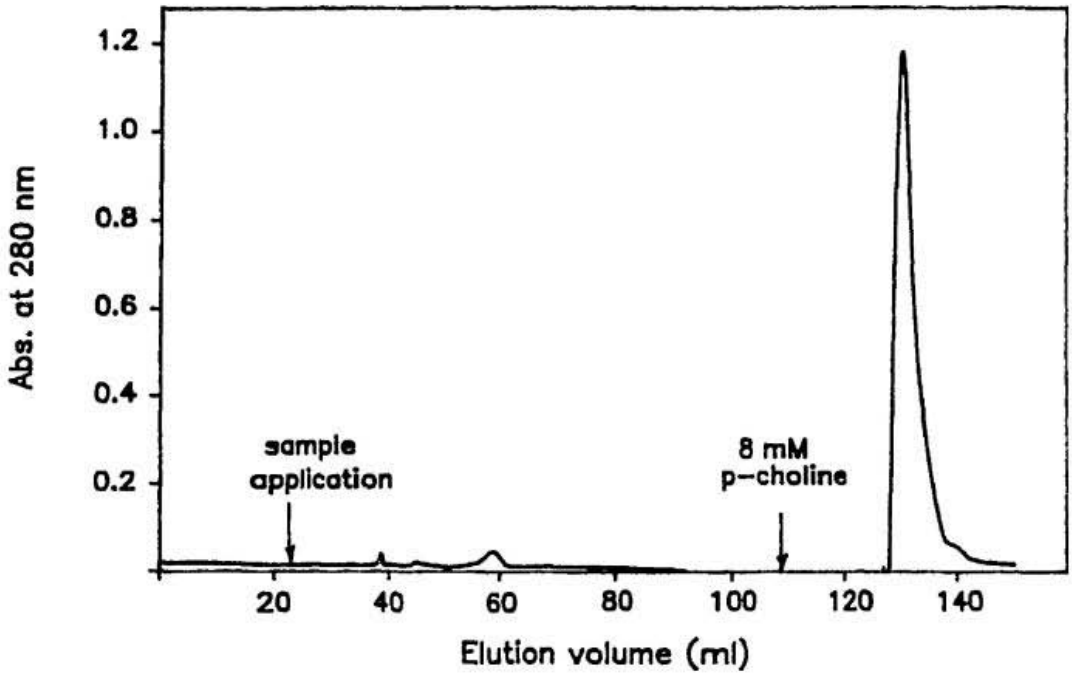
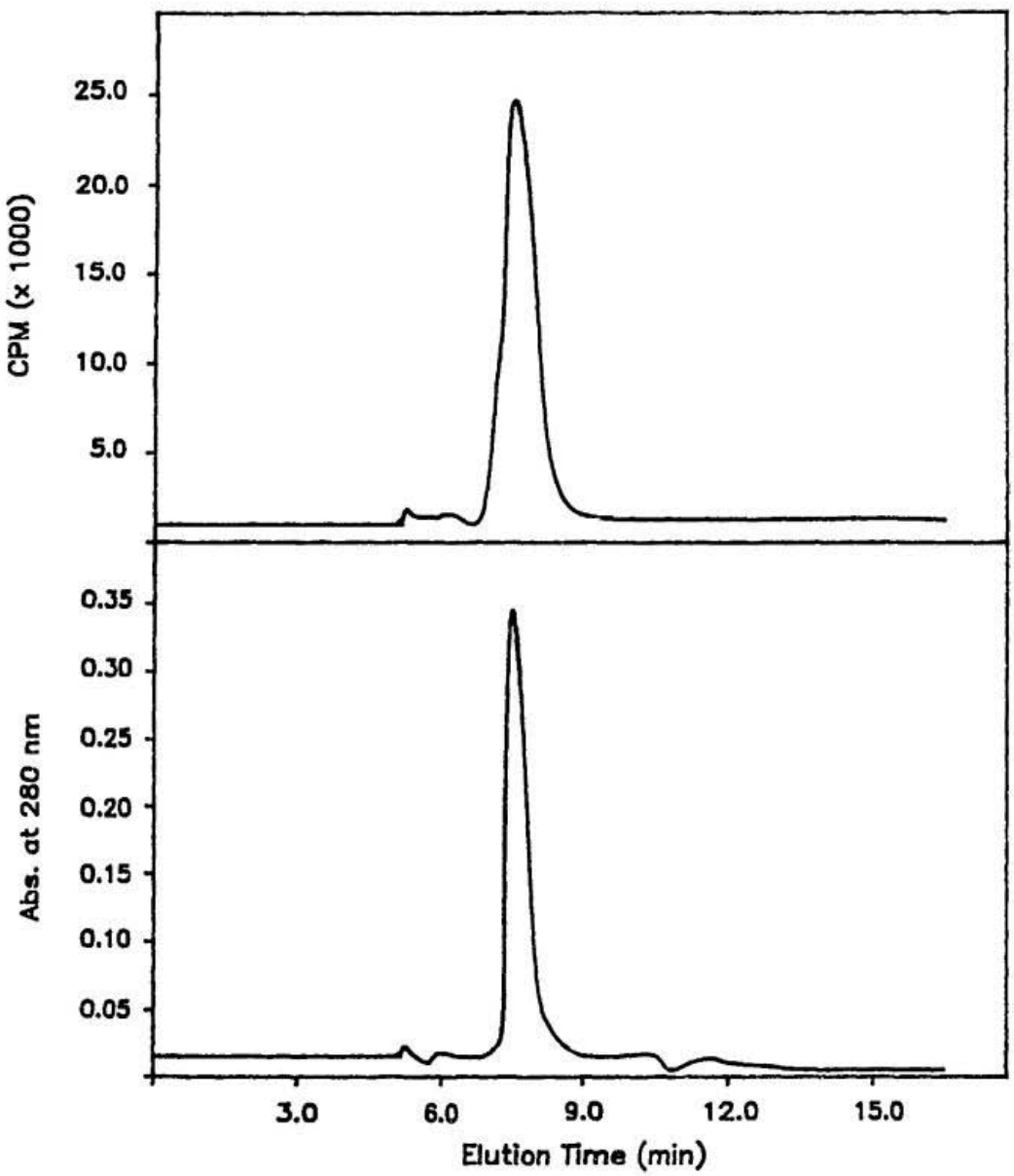


Fig. 6. **Binding of rat asialo CRP to a Sepharose-phenylphosphorylcholine column.** Rat asialo CRP in Tris buffer (20 ml, 0.74 mg/ml) was applied to a Sepharose-phenylphosphorylcholine column (20 x 2 cm) which had been equilibrated with Tris buffer at a flow rate of 1 ml/min. The column was washed extensively with Tris buffer until absorbance at 280 nm of the eluent was less than 0.02. The bound rat asialo CRP was eluted with 8 mM phosphorylcholine in Tris buffer. This graph has been regenerated by digitizing the elution profile with Sigmaplot program 3.1.

Fig. 7. **HPLC analysis of ^{125}I-labeled rat CRP.** ^{125}I-labeled rat CRP in Tris buffer (100 µl, 0.12mg/ml) was applied to a HPLC gel filtration column (300 x 7.5 mm, Bio-Rad) which had been equilibrated with 10 bed volumes of 0.05 M Na_2SO_4-0.02 M NaH_2PO_4 buffer (pH 6.8) at a flow rate of 1.0 ml/min on Perkin-Elmer, Series 4, HPLC system. The column was washed at a flow rate of 1.0 ml/min with the equilibration buffer. The eluent was monitored at 280 nm and also for radioactivity with a Beckman 171 radioisotope detector. This graph has been regenerated by digitizing the elution profiles with Sigmaplot program 3.1.


25.0
20.0
15.0
10.0
5.0
CPM (x 1000)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
Abs. at 280 nm
3.0
6.0
9.0
12.0
15.0
Elution Time (min)

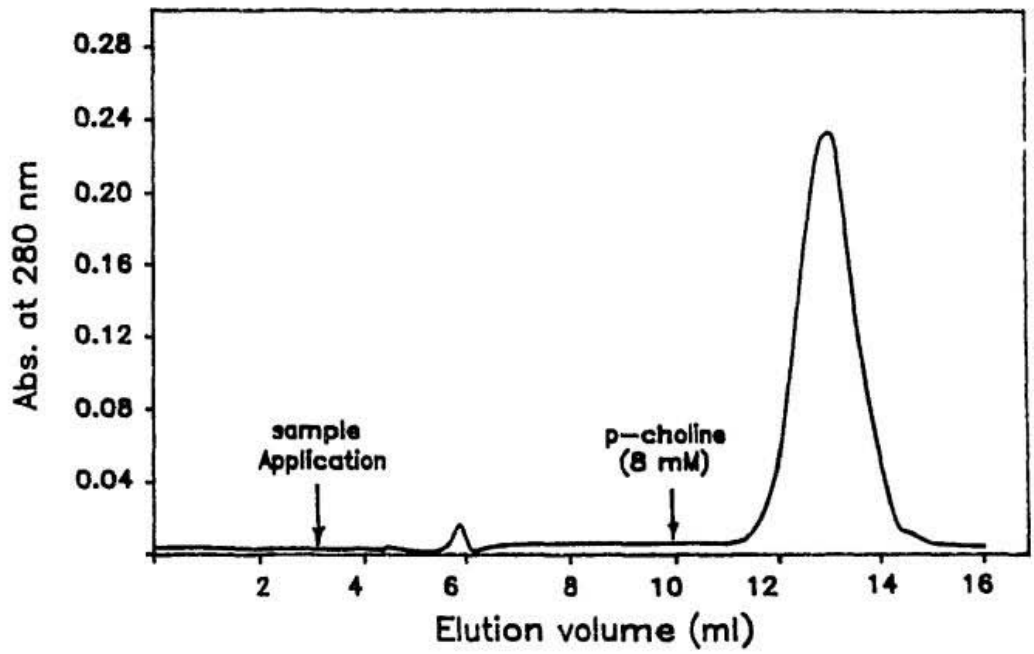


Fig. 8. **Binding of ^{125}I-labeled rat CRP to a Sepharose-phenylphosphorylcholine column.** ^{125}I-labeled rat CRP in Tris buffer (300 µl, 0.12 mg/ml) was applied to a Sepharose-phenylphosphorylcholine column (5 x 0.85 cm) which had been equilibrated with Tris buffer at a flow rate of 0.5 ml/min. The column was washed extensively with Tris buffer until absorbance at 280 nm of the eluent was less than 0.02. The elution buffer was then changed to Tris buffer containing 8 mM phosphorylcholine. This graph has been regenerated by digitizing the elution profile with Sigmaplot program 3.1.

phosphorylcholine-eluted protein peak. Therefore, iodination did not affect the phosphorylcholine binding property of CRP.

C. ESTABLISHMENT OF OPTIMUM CONDITIONS FOR BINDING ASSAYS

1). Requirement of BSA to Prevent the Binding of ^{125}I-labeled Proteins to Glass Tubes

Different concentrations of BSA were tested for their ability to prevent the binding of ^{125}I-labeled proteins to the tubes. As shown in Fig. 9, this binding was dramatically reduced when the tubes were coated with even 0.05% of BSA.

2). Determination of Optimum Cell Concentration and Incubation Time for Binding Assays

The total binding of asialo AGP increased linearly with increasing concentrations of hepatocytes (Fig. 10a). The total binding of rat CRP and rat asialo CRP also showed an increase with increasing cell concentrations (Fig. 10b). A cell concentration of 6.5×10^5 cells/ml was chosen for these proteins, and used in the binding assays.

From the time course of the binding of rat asialo CRP and rat CRP to the hepatocytes (Fig. 11), an incubation time of 90 min was chosen for the binding assays.

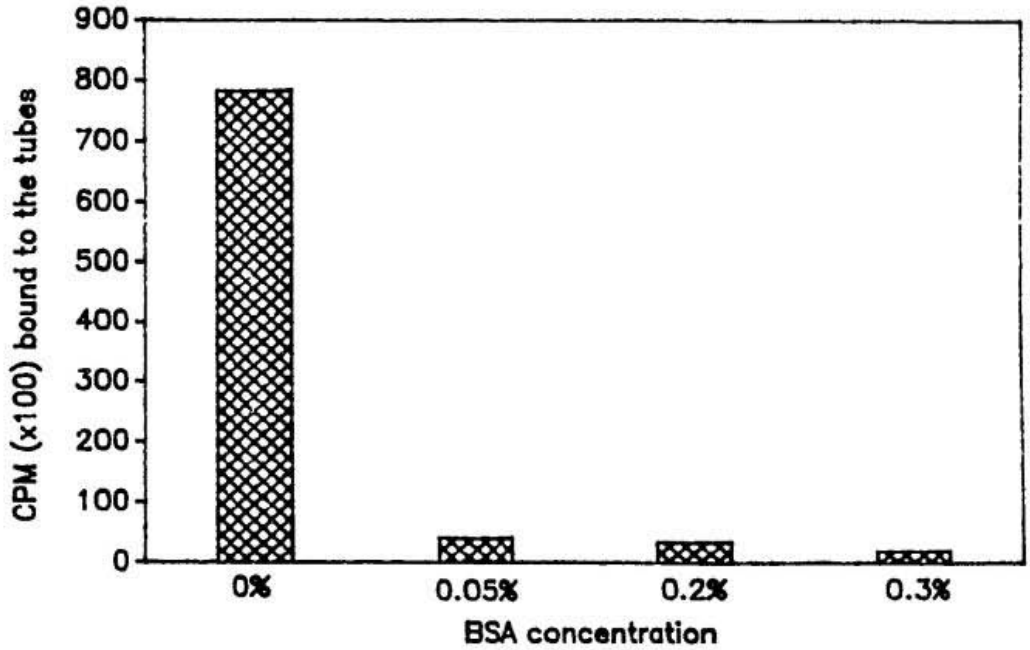
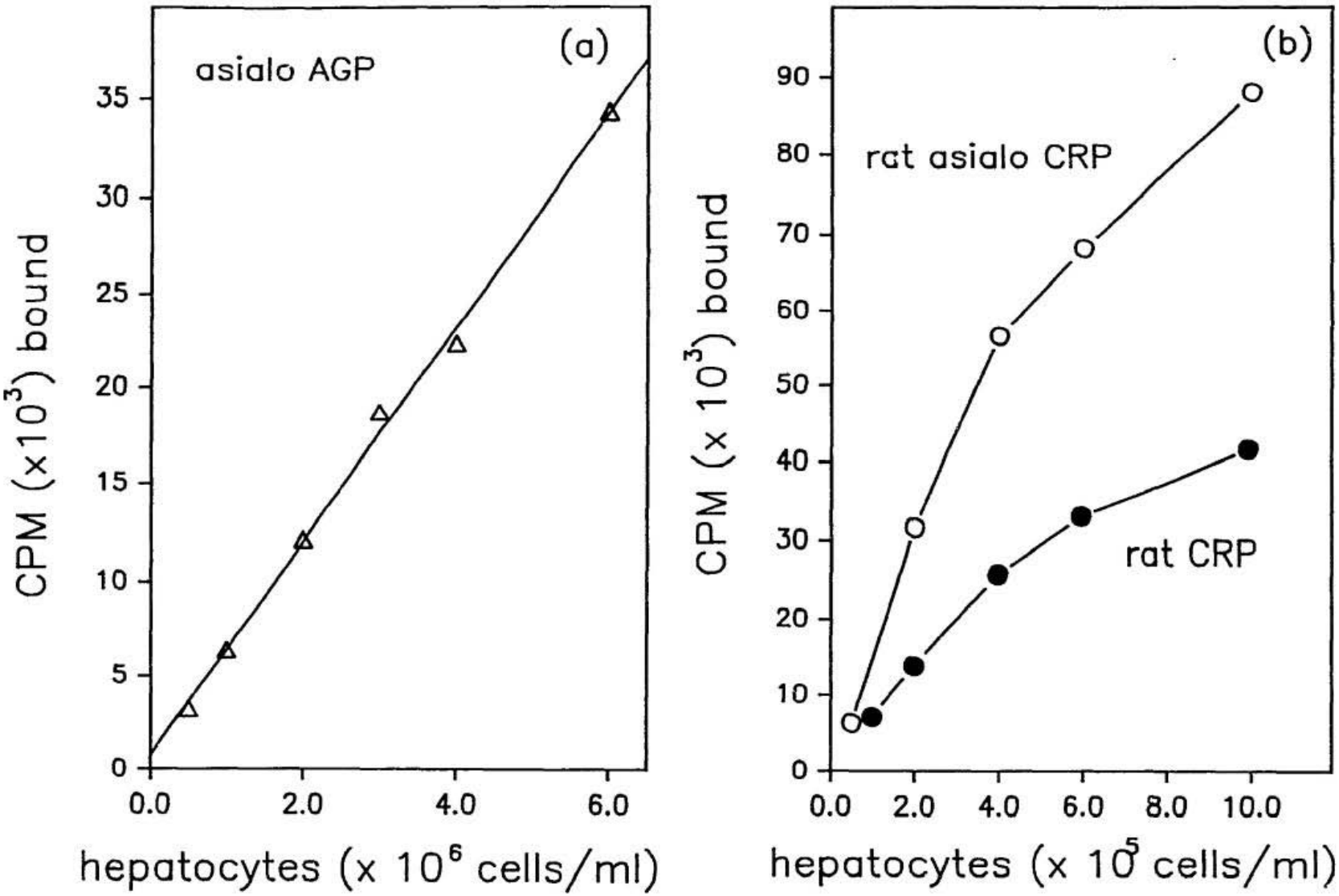


Fig. 9. **Effect of BSA on the binding of ^{125}I-labeled proteins to the tubes.** Glass test tubes (6 x 50 mm) were immersed in 0.05%, 0.2% or 0.3% (w/v) BSA solution in Tris buffer at room temperature. After 1 h, the tubes were taken out and placed upside down for about 30 min to be air-dried. ^{125}I-labeled rat CRP (1 μg in 0.5 ml of Medium/BSA) was then added to the tubes, which were kept at 4°C for 90 min. After being washed three times with Krebs buffer, the tubes were counted for radioactivity in a gamma counter.

Fig. 10 (a & b). **Binding of ^{125}I-labeled asialo AGP, rat asialo CRP and rat CRP to isolated hepatocytes as a function of hepatocyte concentrations.** ^{125}I-labeled asialo AGP (Fig. 10a), rat asialo CRP or rat CRP (Fig. 10b) (1 μg, in Tris buffer) was incubated with increasing concentrations of hepatocytes (85% viable, 88% single cells) in 1 ml of Medium/BSA at 4°C for 60 min. The hepatocytes were then washed and counted for radioactivity as described in chapter 2, pp. 51-52. Each point in the graph is the mean of duplicate determinations and is representative of two separate experiments.


(a)
asialo AGP
CPM (x10^3) bound
0
5
10
15
20
25
30
35
0.0
2.0
4.0
6.0
hepatocytes (x 10^6 cells/ml)
(b)
rat asialo CRP
rat CRP
CPM (x 10^3) bound
0
10
20
30
40
50
60
70
80
90
0.0
2.0
4.0
6.0
8.0
10.0
hepatocytes (x 10^5 cells/ml)

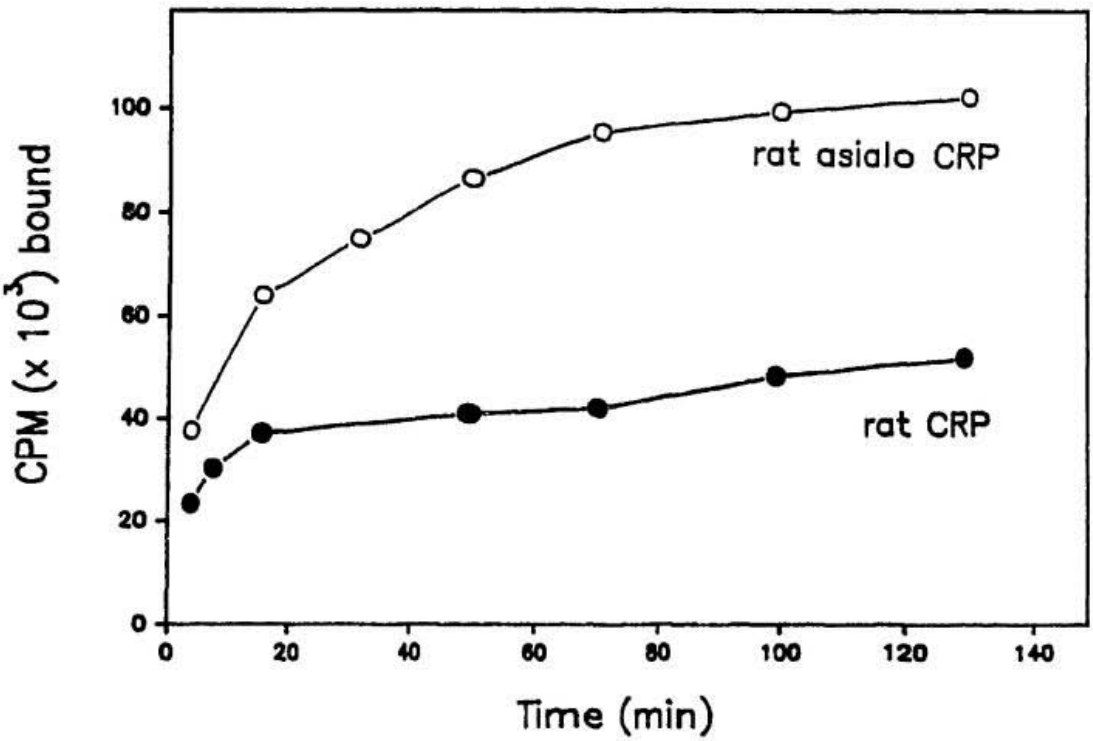


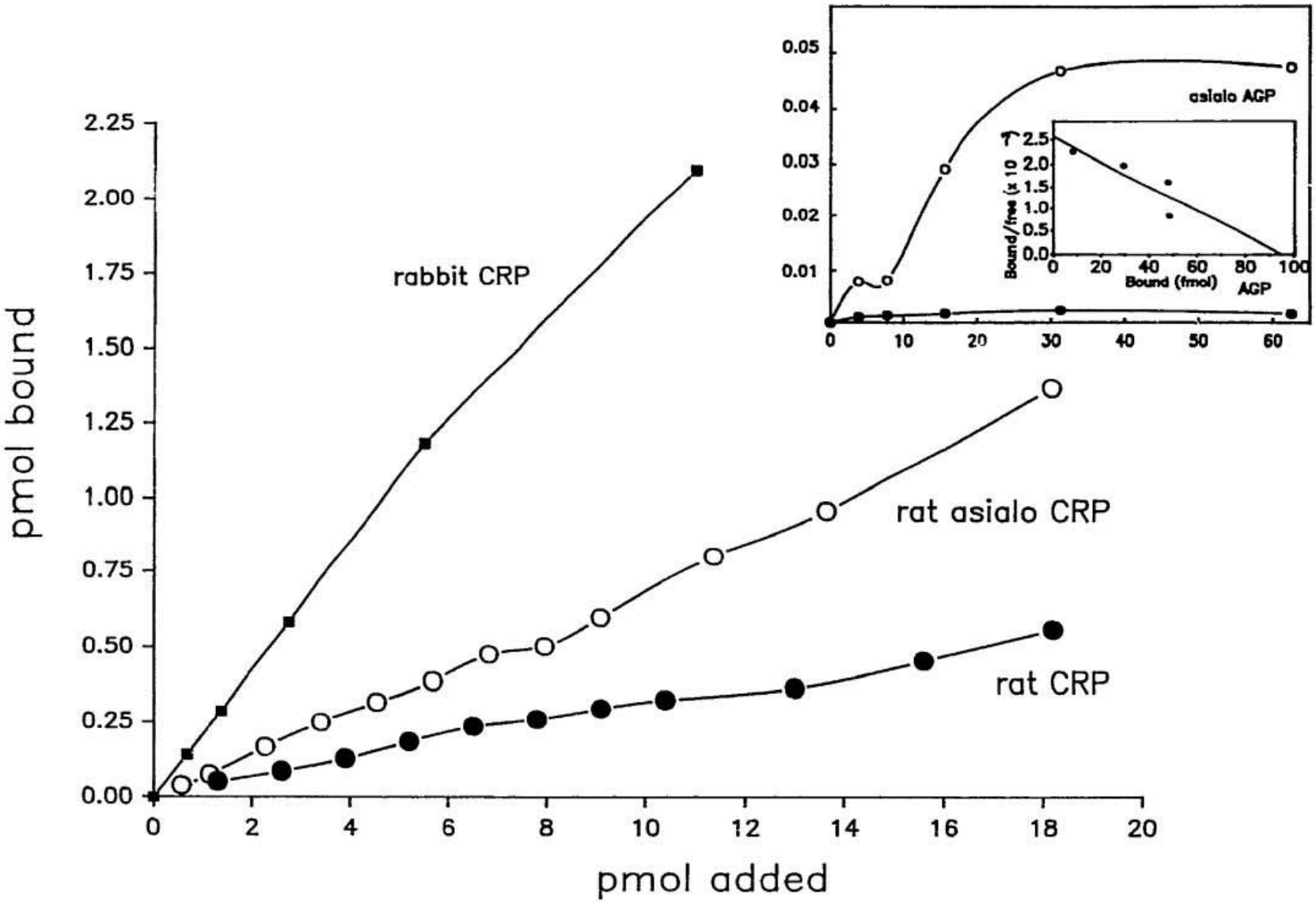
Fig. 11. **Time course of the binding of ^{125}I-labeled rat CRP and rat asialo CRP to isolated hepatocytes.** ^{125}I-labeled rat CRP or rat asialo CRP (1 μg, in Tris buffer) was incubated with 6.5 x 10^5 hepatocytes (85% viable, 85% single cells) in 1 ml of Medium/BSA at 4°C. At the indicated times, the hepatocytes were washed and counted for radioactivity as described in chapter 2, pp. 51-52. Each point in the graph is the mean of duplicate determinations and is representative of two separate experiments.

D. **BINDING OF RAT ASIALO CRP, RAT CRP AND RABBIT CRP TO THE HEPATOCYTES AS A FUNCTION OF PROTEIN CONCENTRATIONS**

The specific binding of asialo AGP to the hepatocytes was saturable, while that of AGP was negligible (Fig. 12, insert). Based on the binding of ^{125}I-labeled asialo AGP to the hepatocytes, the number of the hepatic asialo-glycoprotein receptors on each hepatocyte was calculated to be 8.6×10^4, which was comparable to previously reported values (Table 3). These results are consistent with observations in earlier studies on the binding of asialo AGP to hepatocytes (Ashwell and Harford, 1982).

The amounts of the specific binding of rat asialo CRP, rat CRP and rabbit CRP to the hepatocytes, however, were all much larger compared to that of asialo AGP (Fig. 12). The binding increased linearly within the range of protein concentrations used in this study. Between 4-7% of the protein added bound to the hepatocytes. The total binding of rat CRP (Fig. 13b) and rat asialo CRP (Fig. 13a) was reduced by 70% and 88%, respectively, in the presence of 100-fold excess of unlabeled protein. It is important to note that while the spcific binding of rat asialo CRP to the hepatocytes was about twice that of rat CRP, it was over 30-fold more than that of asialo AGP (Fig. 12).

Fig. 12. **Specific Binding of rat asialo CRP, rat CRP, rabbit CRP, AGP and asialo AGP to isolated hepatocytes as a function of protein concentrations.** Increasing concentrations of ^{125}I-labeled AGP, asialo AGP (insert), rat CRP, rat asialo CRP, or rabbit CRP were incubated with 6.5 x 10^5 hepatocytes (90% viable, 85% single cells) in 1.0 ml of Medium/BSA at 4°C for 90 minutes. The cell bound radioactivity was determined as described in chapter 2, pp. 51-52. The non-specific binding of ^{125}I-labeled AGP and asialo AGP was determined at each point by including 100-fold excess of unlabeled protein in the incubation medium, while the non-specific binding of ^{125}I-labeled rat CRP, rat asialo CRP or rabbit CRP was determined in the presence of 1 mM phosphorylcholine in the incubation medium. In the case of rat asialo CRP, rat CRP and rabbit CRP, each point has been obtained by subtracting the mean of triplicate determinations of non-specific binding from that of the corresponding total binding. The results shown for these proteins are representative of three similar experiments. In the case of asialo AGP and AGP, each point has been obtained by subtracting the mean of duplicate determinations of non-specific binding from that of the corresponding total binding.

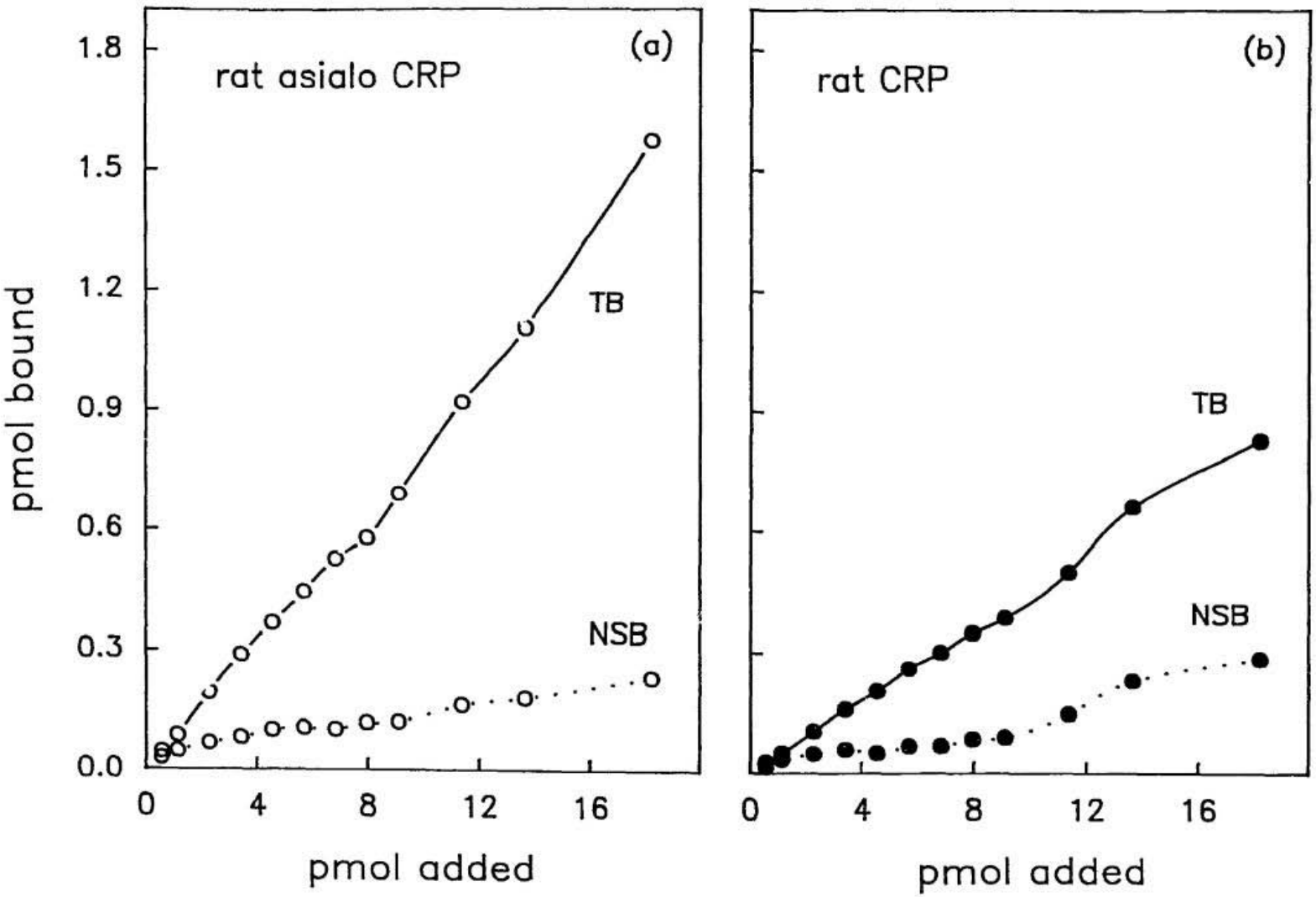

pmol bound
pmol added
rabbit CRP
rat asialo CRP
rat CRP
asialo AGP
Bound/free (x 10⁻³)
Bound (fmol)
AGP

Asialo AGP bound (fmol/6.5 x 10^5 hepatocytes)	93 fmol
Surface content of asialoglycoprotein receptors (number per cell)	8.6 x 10^4/cell
Reported surface content of asialoglycoprotein receptors* (number per cell)	5-50 x 10^4/cell

Table 3. **Surface content of asialoglycoprotein receptors on isolated hepatocytes.** The binding of ^{125}I-labeled asialo AGP to isolated hepatocytes were carried out as described in the legend of Fig. 12. The number of asialoglycoprotein receptors on the isolated hepatocytes was obtained by Scatchard analysis of the binding data (Fig. 12, insert), and based on the assumption that the specific binding of asialo AGP represents the binding to the hepatic asialoglycoprotein receptor only, and one molecule of asialo AGP occupies one receptor.

*Quoted from Weigel and Oka (1983).

Fig. 13 (a & b). **Binding of ^{125}I-labeled rat asialo CRP and rat CRP to isolated hepatocytes as a function of protein concentrations.** The experimental details are the same as described in the legend of Fig. 12, except that the non-specific binding of ^{125}I-labeled rat asialo CRP and rat CRP was determined by including 100-fold excess of unlabeled protein in the incubation medium. Each point in the graph is the mean of triplicate determinations and is representative of two similar experiments. (TB: total binding; NSB: non-specific binding).


(a)
rat asialo CRP
TB
NSB
pmol bound
pmol added
0.0
0.3
0.6
0.9
1.2
1.5
1.8
0
4
8
12
16
(b)
rat CRP
TB
NSB
pmol added
0
4
8
12
16

E. EFFECT OF GalNAc AND PHOSPHORYLCHOLINE ON THE BINDING OF RAT ASIALO CRP, RAT CRP AND RABBIT CRP TO THE HEPATOCYTES

In order to characterize the binding of rat asialo CRP, rat CRP and rabbit CRP to the hepatocytes, the effect of GalNAc and phosphorylcholine on their binding was examined. GalNAc is a known inhibitor of the binding of asialoglycoproteins to the hepatic asialoglycoprotein receptor (Ashwell and Harford, 1982). Phosphorylcholine is the ligand that CRP binds, and has been shown to be involved in most CRP related reactions (Tomasz, 1967; Volanakis and Kaplan, 1971).

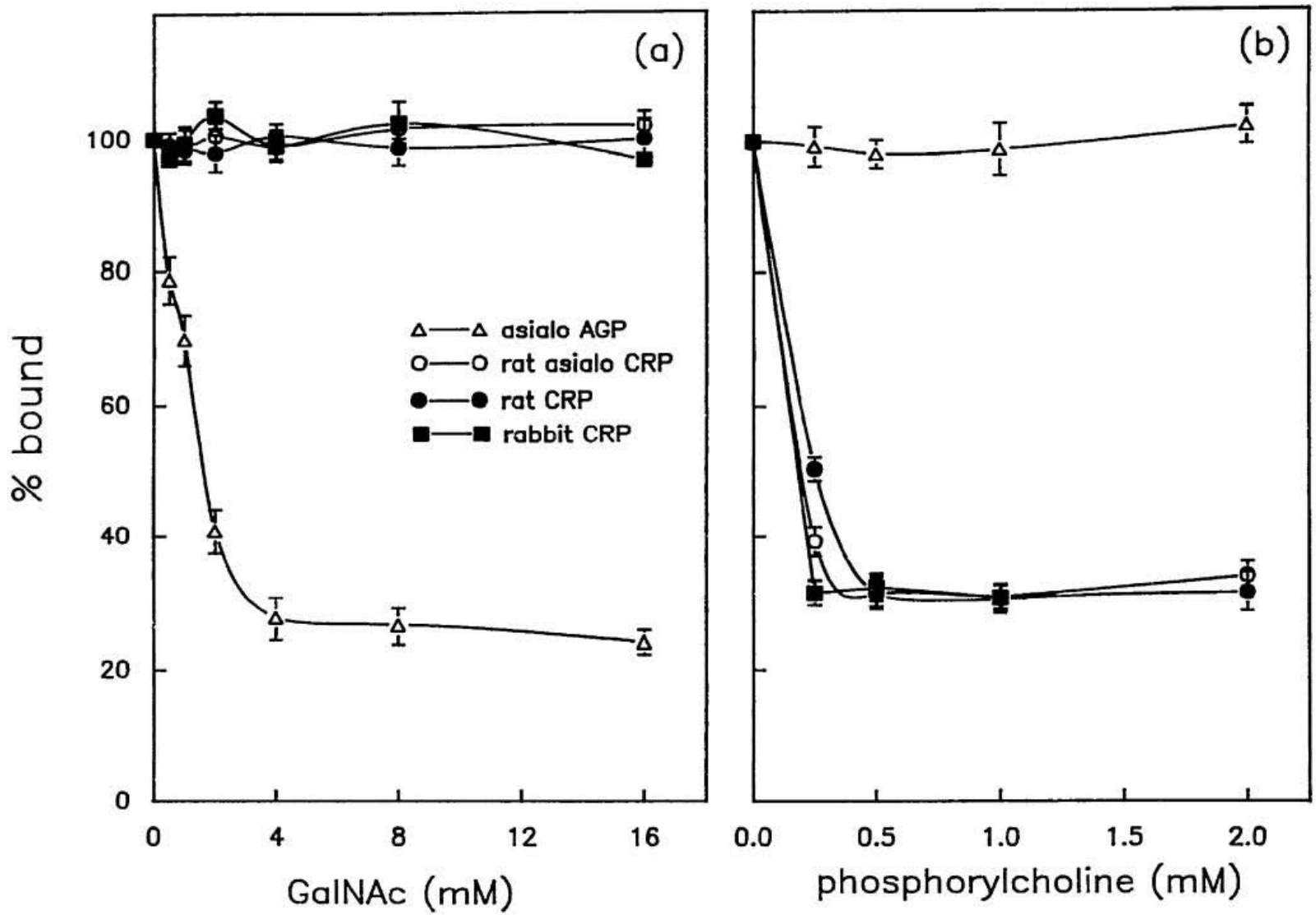
The binding of asialo AGP to the hepatocytes was inhibited by GalNAc in a dose-dependent manner (Fig. 14a), whereas the binding of rat asialo CRP, rat CRP and rabbit CRP to the hepatocytes did not change in the presence of GalNAc (Fig. 14a). In contrast, phosphorylcholine inhibited (about 75%) the binding of rat asialo CRP, rat CRP and rabbit CRP to the hepatocytes, but had not effect on the binding of asialo AGP (Fig. 14b).

F. COMPETITIVE INHIBITION STUDIES ON THE BINDING OF RAT ASIALO CRP, RAT CRP AND ASIALO AGP TO THE HEPATOCYTES

Competitive inhibition is based on the assumption that if two proteins interact with the same binding site, they compete with each other for occupying that binding site.

When the hepatocytes were pre-incubated with increasing

Fig. 14. **Effect of GalNAc and phosphorylcholine on the binding of rat CRP, rat asialo CRP, rabbit CRP and asialo AGP to isolated hepatocytes.** ^{125}I-labeled rat CRP, rat asialo CRP, rabbit CRP or asialo AGP (1 μg, in Tris buffer) was incubated with 6.5 x 10^5 hepatocytes (85% viable, 85% single cells) in 1 ml of Medium/BSA at 4°C for 90 min. Increasing concentrations of GalNAc (Fig. 14a) or phosphorylcholine (Fig. 14b) were then added to the incubation medium and the cell suspensions were incubated for another 15 min. Cell bound radioactivity was determined as described in chapter 2, pp. 51-52. The amount of ^{125}I-labeled protein bound in the presence of different concentrations of GalNAc or phosphorylcholine has been expressed as the percentage of that in the absence of GalNAc and phosphorylcholine. Each point in this graph is the mean ± SEM of three separate experiments.


(a)
(b)
% bound
100
80
60
40
20
0
0
4
8
12
16
GalNAc (mM)
asialo AGP
rat asialo CRP
rat CRP
rabbit CRP
0.0
0.5
1.0
1.5
2.0
phosphorylcholine (mM)

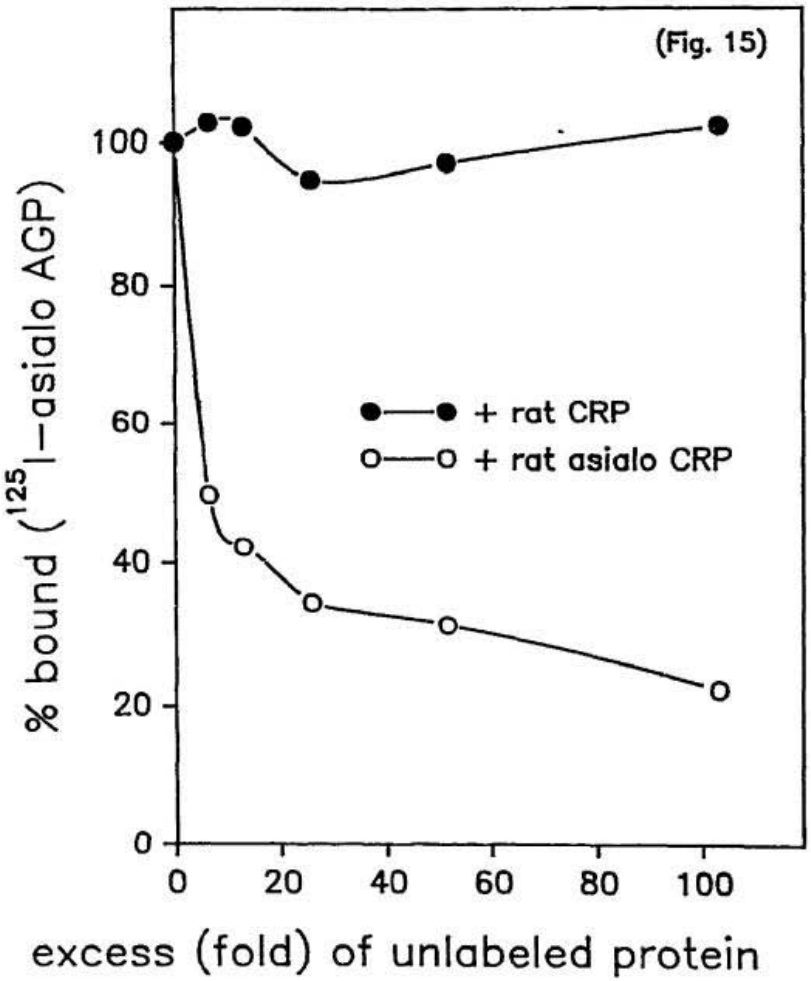
concentrations of rat asialo CRP, the binding of ^{125}I-labeled asialo AGP to the hepatocytes decreased by up to 80% in a dose-dependent manner (Fig. 15). In contrast, pre-incubating the hepatocytes with rat CRP had no effect on the binding of ^{125}I-labeled asialo AGP to the hepatocytes (Fig. 15). These results suggest that rat asialo CRP binds to the hepatic asialoglycoprotein receptor.

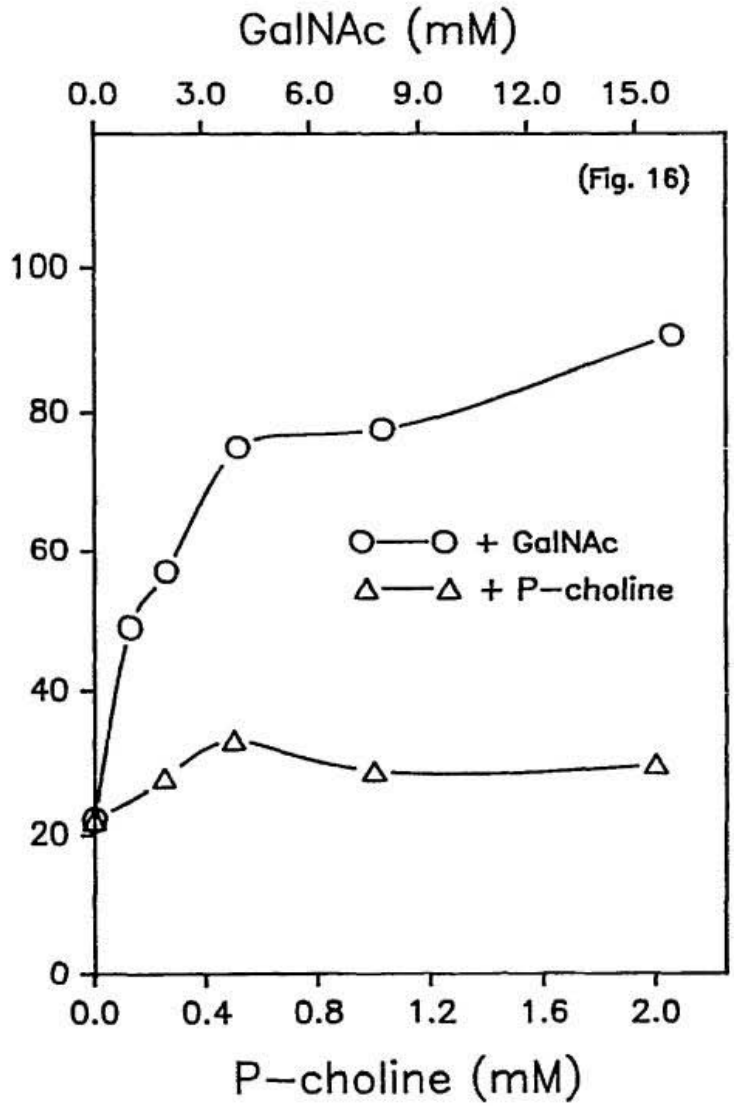
In another set of experiments, hepatocytes were pre-incubated with rat asialo CRP and increasing concentrations of either GalNAc or phosphorylcholine. After the cells had been washed, ^{125}I-labeled asialo AGP was added, and the cell suspension was further incubated. The results showed that GalNAc restored the binding of ^{125}I-labeled asialo AGP to the hepatocytes pre-bound with rat asialo CRP (Fig. 16). This indicates that GalNAc releases rat asialo CRP from the hepatic asialoglycoprotein receptor, and confirms the involvement of the exposed galactose residues of rat asialo CRP in the binding of this protein to the hepatic asialo glycoprotein receptor. In contrast, phosphorylcholine did not increase the binding of ^{125}I-labeled asialo AGP to the hepatocytes pre-bound with rat asialo CRP (Fig. 16). This shows that, although phosphorylcholine releases more than 75% of rat asialo CRP from the hepatocytes (Fig. 14b), it did not release any rat asialo CRP bound to the hepatic asialoglycoprotein receptors.

When the binding of ^{125}I-labeled rat asialo CRP to the

Fig. 15. **Competitive inhibition of the binding of ^{125}I-labeled asialo AGP to isolated hepatocytes by rat asialo CRP or rat CRP.** Increasing amounts of rat asialo CRP or rat CRP were incubated with 6.5 x 10^5 hepatocytes (90% viable, 85% single cells) in 1 ml of Medium/BSA at 4°C. After 60 min, 1 μg of ^{125}I-labeled asialo AGP (in Tris buffer) was added to each tube. The cell suspensions were incubated for another 90 min at 4°C. Cell bound radioactivity was then determined as described in chapter 2, pp. 51-52. The amount of ^{125}I-labeled asialo AGP bound to the cells in the presence of different concentrations of either rat asialo CRP or rat CRP has been expressed as the percentage of that in the absence of rat asialo CRP or rat CRP. Each point in this graph is the mean of duplicate determinations and is representative of two similar experiments.

Fig. 16. **Effect of GalNAc and phosphorylcholine on the competitive inhibition of the binding of ^{125}I-labeled asialo AGP by rat asialo CRP.** Rat asialo CRP (300 μg in Tris buffer) was incubated at 4°C for 60 min with 6.5 x 10^5 hepatocytes (90% viable, 85% single cells) in 2 ml of Medium/BSA in the presence of 8 Mm GalNAc or 1 mM phosphorylcholine. The cells were then washed twice with Medium/BSA and re-suspended in 1 ml of Medium/BSA. ^{125}I-labeled asialo AGP (1 μg in Tris buffer) was added to each tube, and the cell suspensions were incubated for another 90 min at 4°C. The cell bound radioactivity was determined as described in chapter 2, pp. 51-52. The amount of ^{125}I-labeled asialo AGP bound to the cells after the treatment with different concentrations of either GalNAc or phosphorylcholine has been expressed as the percentage of that in the absence of rat asialo CRP, GalNAc and phosphorylcholine. Each point in this graph is the mean of duplicate determinations and is representative of two separate experiments.


(Fig. 15)
% bound (^{125}I–asialo AGP)
100
80
60
40
20
0
0
20
40
60
80
100
+ rat CRP
+ rat asialo CRP
excess (fold) of unlabeled protein


GalNAc (mM)
0.0
3.0
6.0
9.0
12.0
15.0
(Fig. 16)
100
80
60
40
20
0
0.0
0.4
0.8
1.2
1.6
2.0
+ GalNAc
+ P–choline
P–choline (mM)

hepatocytes was carried out in the presence of increasing concentrations of rat CRP, there was a dose-dependent decrease of the binding of ^{125}I-labeled rat asialo CRP to the hepatocytes (Fig. 17). On the other hand, AGP had no effect on the binding of ^{125}I-labeled asialo AGP to the hepatocytes (Fig. 17). This indicates that, in addition to its binding to the hepatic asialoglycoprotein receptor, rat asialo CRP shares with rat CRP a common binding site on the hepatocytes, which is different from the hepatic asialoglycoprotein receptor.

G. BINDING OF RAT ASIALO CRP TO ISOLATED HEPATOCYTES WITH DIFFERENT VIABILITY

It has been suggested that the binding of CRP to cell membranes requires a disruption of the structure of the membranes (Volanakis and Wirtz, 1979; Narkates and Volanakis, 1982). In order to provide a rational explanation for the avid binding of CRP to the hepatocytes observed in this study, hepatocytes with different viability were used in the binding assays. As shown in Fig. 18, there was an increase in the binding of rat asialo CRP to hepatocytes with decreasing viability.

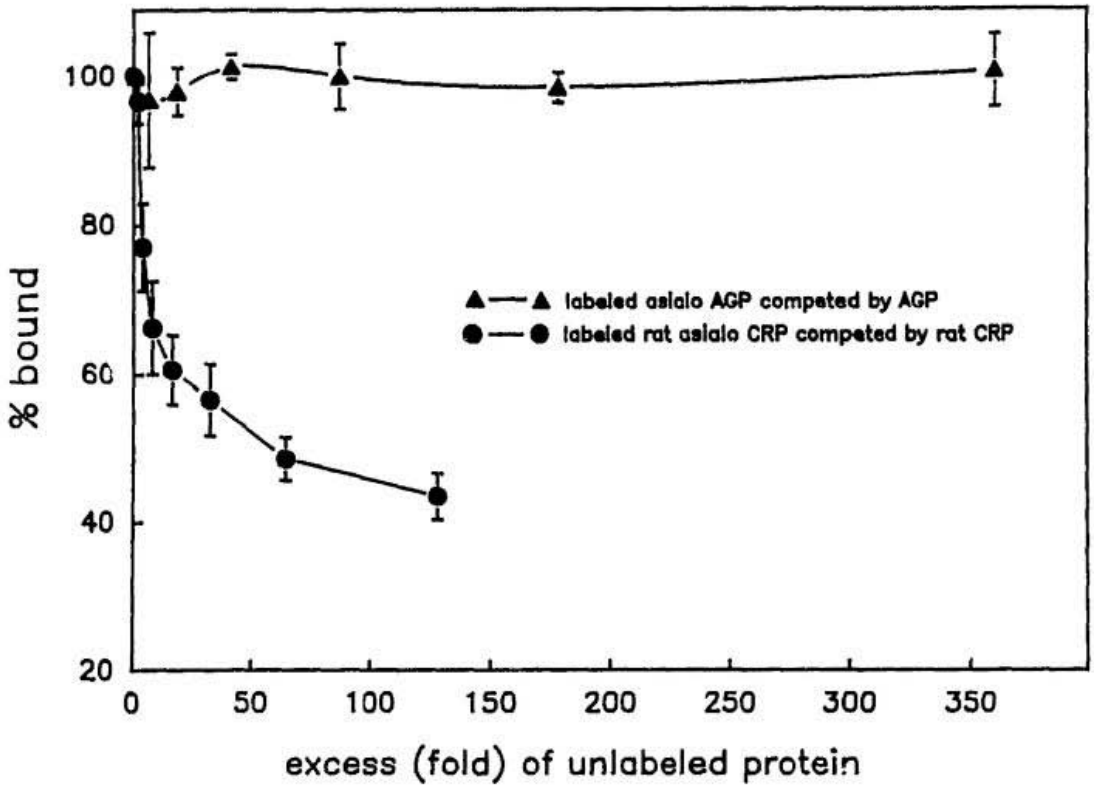


Fig. 17. **Competitive Inhibition of the binding of ^{125}I-labeled rat asialo CRP and asialo AGP to isolated hepatocytes by rat CRP and AGP.** ^{125}I-labeled rat asialo CRP or asialo AGP was incubated with 6.5 x 10^5 hepatocytes (85% viable, 85% single cells) in 1 ml of Medium/BSA at 4°C for 90 min in the presence of increasing amounts of rat CRP or AGP. Cell bound radioactivity was determined as described in chapter 2, pp. 51-52. The amount of ^{125}I-labeled asialo AGP or rat asialo CRP bound to the cells in the presence of different concentrations of unlabeled proteins has been expressed as the percentage of that in the absence of unlabeled proteins. Each point in the graph is the mean ± 2 SD of two separate experiments.

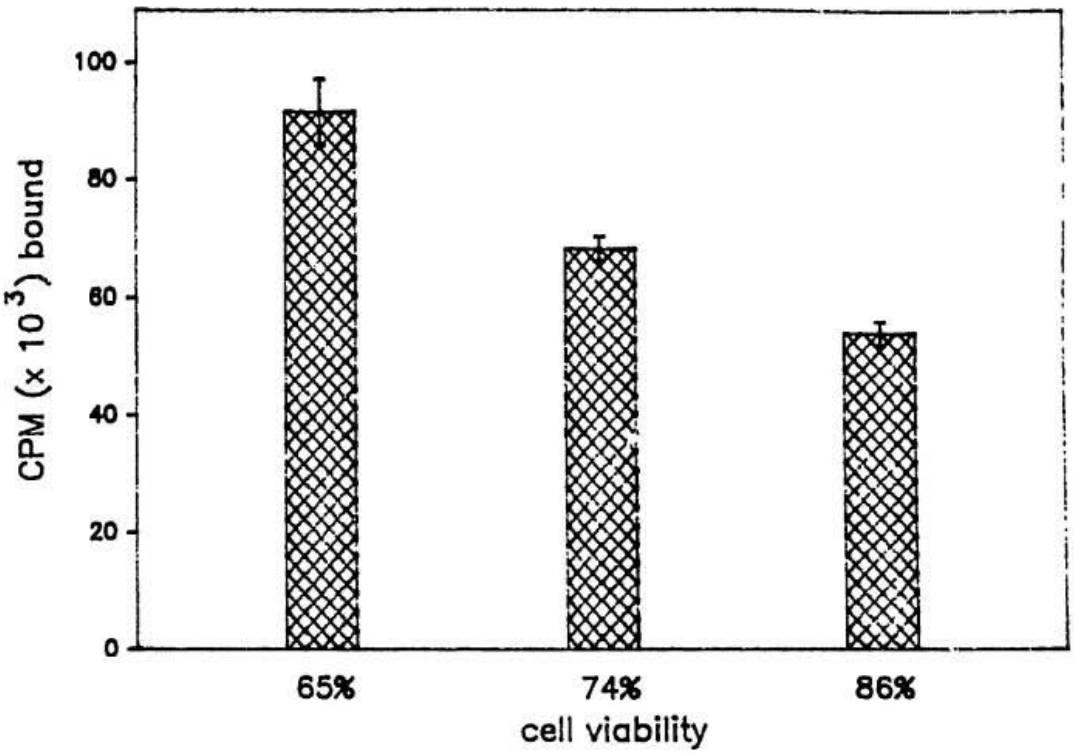


Fig. 18. **Binding of ^{125}I-labeled rat asialo CRP to isolated hepatocytes with different viability.** ^{125}I-labeled rat asialo CRP (2 μg, in Tris buffer) was incubated for 70 min with 1.0 x 10^6 hepatocytes with 86%, 74% or 65% viability in 1.0 ml of Medium/BSA. The cell bound radioactivity was then determined as described in chapter 2, pp. 51-52. The data shown in this graph is the mean ± SEM of triplicate determinations.

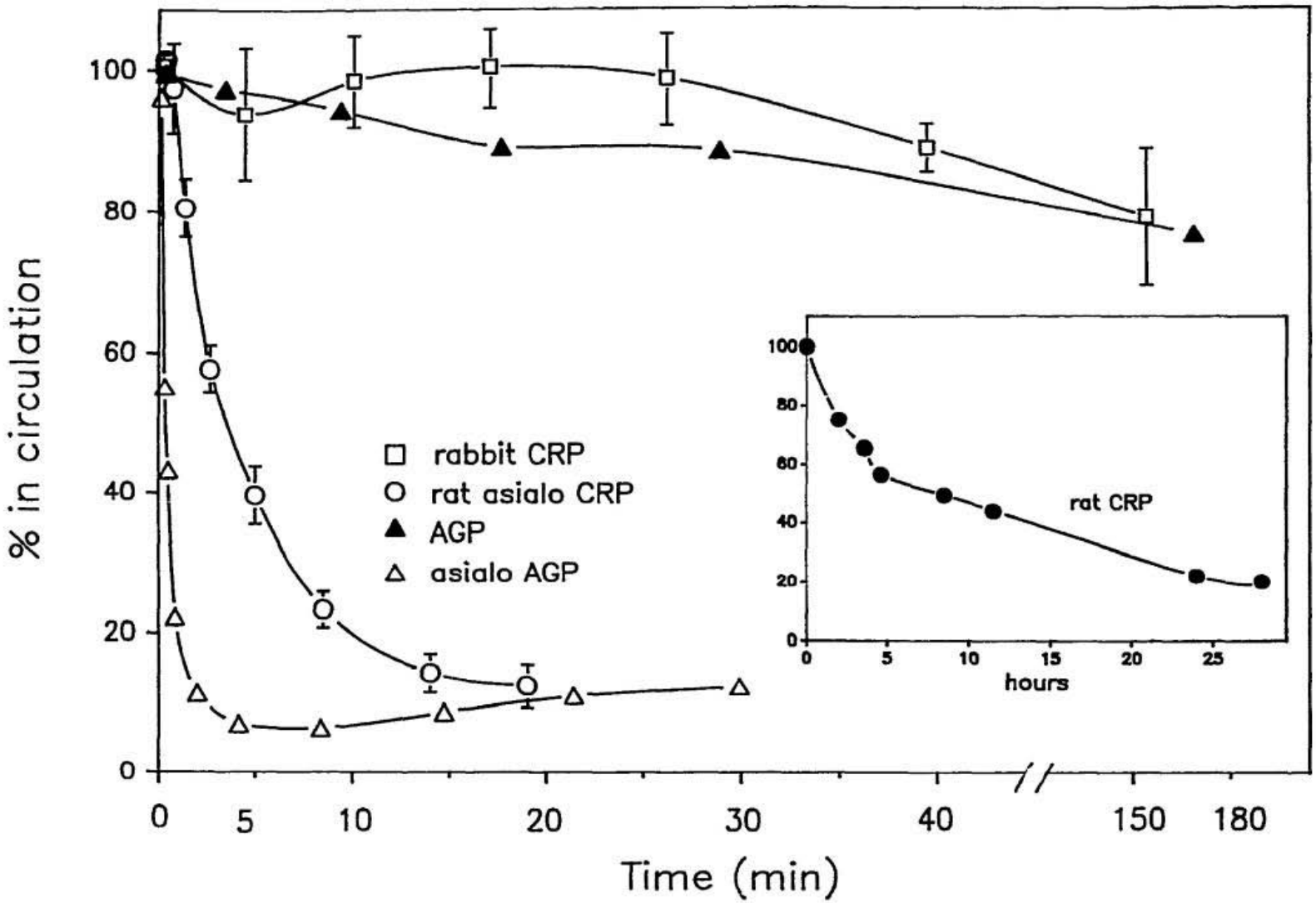
Section II. CLEARANCE OF RAT ASIALO CRP, RAT CRP AND RABBIT CRP IN VIVO AND BY PERFUSED LIVER

In section I, the binding of rat asialo CRP, rat CRP and rabbit CRP to isolated hepatocytes was studied. Large amounts of binding of these CRP to the hepatocytes were observed. This binding was inhibited by phosphorylcholine. The binding of rat asialo CRP to the hepatic asialoglycoprotein receptor could be shown only indirectly. In this section, the *in vivo* clearance of CRP was studied in order to determine a). if the phosphorylcholine binding domain of CRP is involved in CRP's *in vivo* clearance; b). if rat asialo CRP is cleared through Ashwell's pathway; and c). if non-hepatic tissues are involved in the clearance of these proteins.

A. CLEARANCE OF ^{125}I-LABELED RAT ASIALO CRP, RAT CRP AND RABBIT CRP IN RAT

Rat asialo CRP and asialo AGP were rapidly removed from circulation (Fig. 19). Four min after the injection, about 50% of rat asialo CRP and more than 90% of asialo AGP were cleared from circulation (Fig. 19). In contrast, the radioactivity associated with ^{125}I-labeled AGP and rabbit CRP remained in circulation for over 3 hours (Fig. 19). The clearance of rat CRP was examined for 28 hours. The half-life obtained was about 7.8 hour, which is much longer than

Fig. 19. **Clearance of rat asialo CRP, and rat CRP and rabbit CRP from circulation.** ^{125}I-labeled protein sample (10-15 μg, 8-15 x 10^6 CPM, in 250 μl of Tris buffer) was injected through the tail vein. The radioactivity in blood samples was determined as described in chapter 2, pp. 47-48. The amount of radioactivity in blood samples collected at different times has been expressed as the percentage of that in the first blood sample. In the case of rat asialo CRP, each point is the mean ± SEM of three separate experiments. In the case of rabbit CRP, each point is the mean ± 2 SD of two separate experiments. In the case of rat CRP, the experiments were carried out in 5 rats, which were sacrificed at different times. Each point in the graph represents data obtained from one rat.


% in circulation
100
80
60
40
20
0
0
5
10
20
30
40
150
180
Time (min)
rabbit CRP
rat asialo CRP
AGP
asialo AGP
rat CRP
hours
0
5
10
15
20
25

its desialylated derivative (Fig. 19, insert).

B. COMPETITIVE INHIBITION OF THE IN VIVO CLEARANCE OF RAT ASIALO CRP BY ASIALO AGP, ASIALO-FETUIN AND AGP

When ^{125}I-labeled rat asialo CRP was injected with increasing amounts of asialo AGP, a dose-dependent inhibition of the clearance of ^{125}I-labeled rat asialo CRP was observed (Fig. 20). A 60-fold excess of asialo AGP over ^{125}I-labeled rat asialo CRP partially inhibited the clearance of ^{125}I-labeled rat asialo CRP, while a 300-fold excess of asialo AGP completely inhibited the clearance. Similarly, the addition of a 300-fold excess of asialo-fetuin also resulted in a nearly complete inhibition of the clearance of ^{125}I-labeled rat asialo CRP (Fig. 21). In contrast, over 1,000-fold excess of AGP had no effect on the clearance of ^{125}I-labeled rat asialo CRP (Fig. 21).

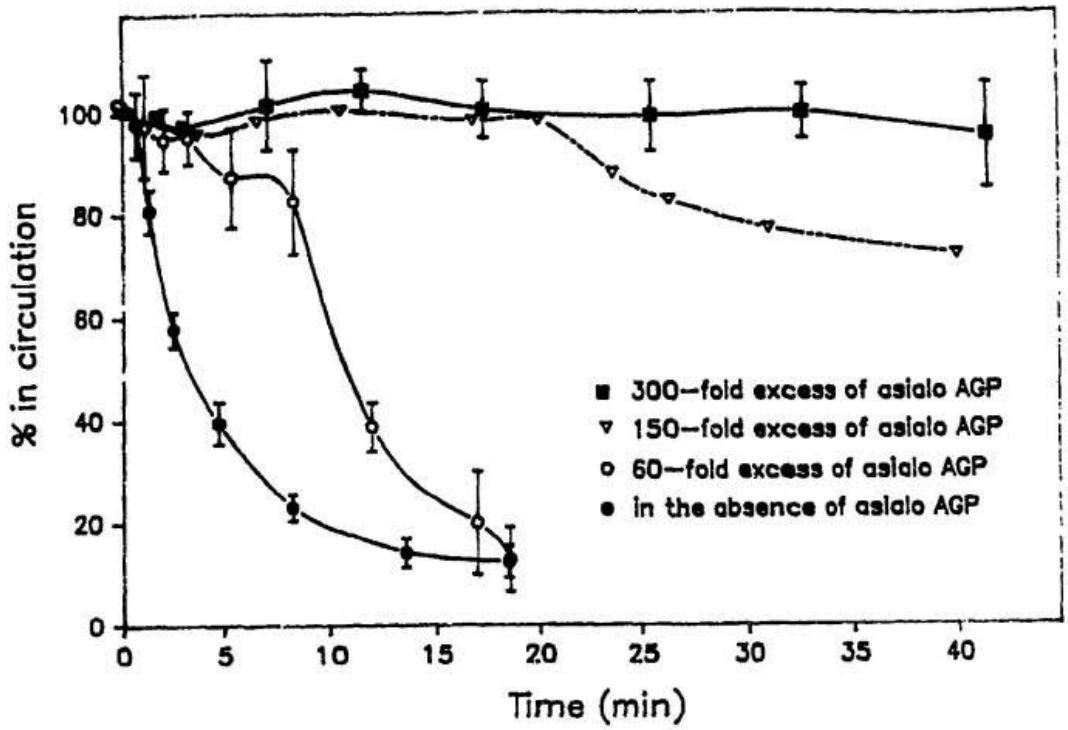
C. DISTRIBUTION OF RADIOACTIVITY IN TISSUES FOLLOWING THE CLEARANCE OF ^{125}I-LABELED RAT ASIALO CRP, RAT CRP AND RABBIT CRP

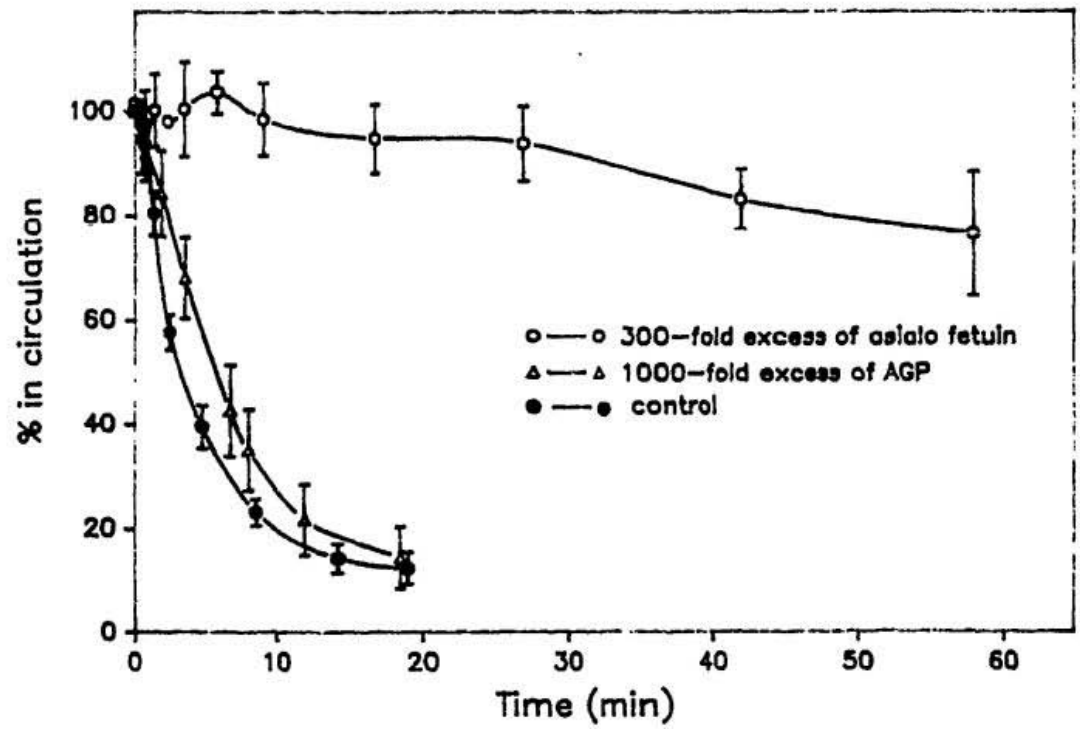
The distribution of radioactivity in liver, lungs, kidney, spleen, stomach, thymus, heart, bone marrow, pancreas and intestine was examined after the injection of ^{125}I-labeled rat asialo CRP, asialo AGP, rat CRP and rabbit CRP.

In the case of ^{125}I-labeled rat asialo CRP, the highest

Fig. 20. **Competitive inhibition of the clearance of ^{125}I-labeled rat asialo CRP by asialo AGP.** ^{125}I-labeled rat asialo CRP (15 μg, 1.1 x 10^7 CPM, in 250 μl of Tris buffer) was injected into the tail vein along with 300 μg (60-fold excess), 750 μg (150-fold excess) or 1,500 μg (300-fold excess) of asialo AGP. The radioactivity in blood samples collected at different time was determined as described in chapter 2, pp. 47-48. The percentage of radioactive label in circulation at different times was calculated as described in the legend of Fig. 19. For the clearance of rat asialo CRP in the absence of asialo AGP, each point in the graph is the mean ± SEM of three separate experiments. For the clearance of rat asialo CRP in the presence of 60-fold or 300-fold excess of asialo AGP, each point is the mean ± 2 SD of two separate experiments.

Fig. 21. **Competitive inhibition of the clearance of ^{125}I-labeled rat asialo CRP by asialo-fetuin or AGP.** ^{125}I-labeled rat ds-CRP (15 μg, 1.1 x 10^7 CPM, in 250 μl of Tris buffer) was injected into the tail vein along with 1.8 mg of asialo-fetuin (300-fold excess) or 5 mg of AGP (1000-fold excess). The radioactivity in blood samples collected at different time was determined as described in chapter 2, pp. 47-48. The percentage of radioactive label in circulation at different times was calculated as described in the legend of Fig. 19. Control refers to the clearance of ^{125}I-labeled rat asialo CRP in the absence of asialo-fetuin or AGP. In the case of control group, each point in the graph is the mean ± SEM of three separate experiments. For the clearance of rat asialo CRP in the presence of AGP or asialo-fetuin, each point in the graph is the mean ± 2 SD of two separate experiments.


% in circulation
Time (min)
300–fold excess of asialo AGP
150–fold excess of asialo AGP
60–fold excess of asialo AGP
in the absence of asialo AGP


% in circulation
Time (min)
300–fold excess of asialo fetuin
1000–fold excess of AGP
control

radioactivity (per gram tissue) was found in the liver (Fig. 22). The recovery of radioactivity associated with rat asialo CRP, rat CRP, rabbit CRP and asialo AGP is listed in Table 4. The high recovery of the radioactivity in the liver associated with ^{125}I-labeled rat asialo CRP was similar to that associated with ^{125}I-labeled asialo AGP. On the other hand, only small amounts of radioactivity associated with ^{125}I-labeled rat CRP and rabbit CRP were recovered in the liver. As shown in Fig. 23a, the radioactivity in the liver decreased rapidly with time.

Some radioactivity associated with ^{125}I-labeled rat CRP, rat asialo CRP and rabbit CRP was also recovered in the lungs (Table 4 & Fig. 23b). Here, the radioactivity did not change with time for up to 60 min. However, after 180 min, there was a reduction of radioactivity in the lungs in the case of ^{125}I-labeled rat asialo CRP and rat CRP (rabbit CRP was not examined at 180 min). In contrast, no radioactivity associated with either ^{125}I-labeled AGP or asialo AGP was detected in the lungs (Table 4 & Fig. 23b).

In the competition inhibition experiments of the clearance of ^{125}I-labeled rat asialo CRP by asialo AGP (Fig. 20), the recovery of radioactivity in liver and lungs was examined. In the presence of increasing concentrations of asialo AGP, the recovery of radioactivity associated with ^{125}I-labeled rat asialo CRP in liver decreased (Fig. 24). At the same time, the radioactivity associated with ^{125}I-labeled

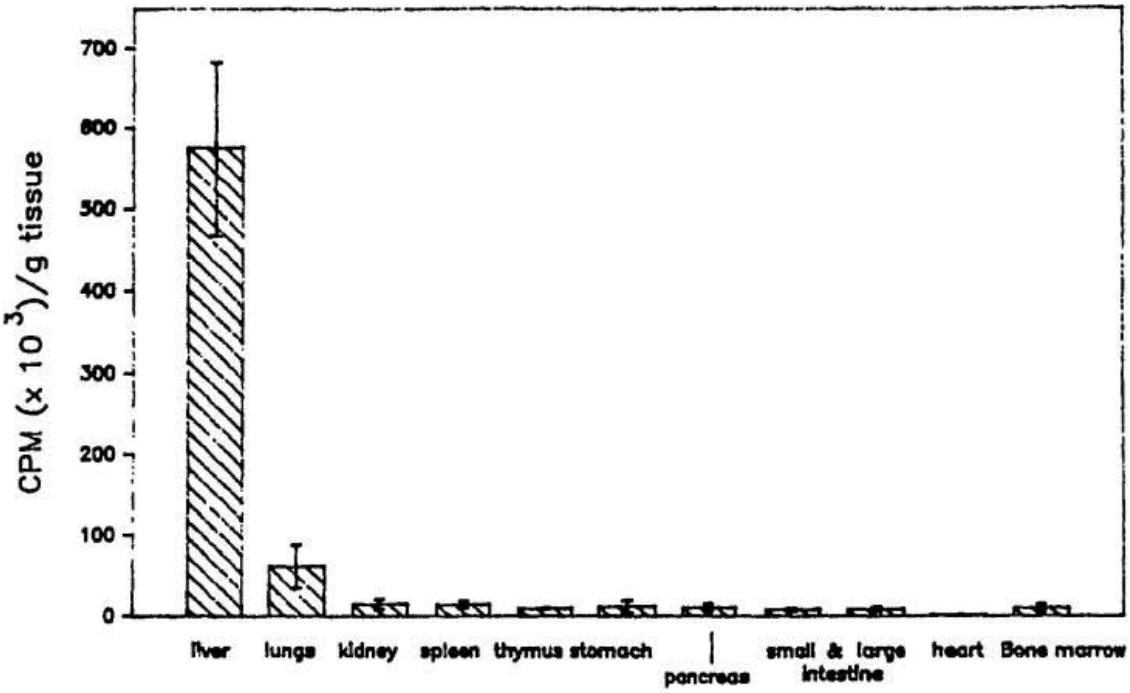
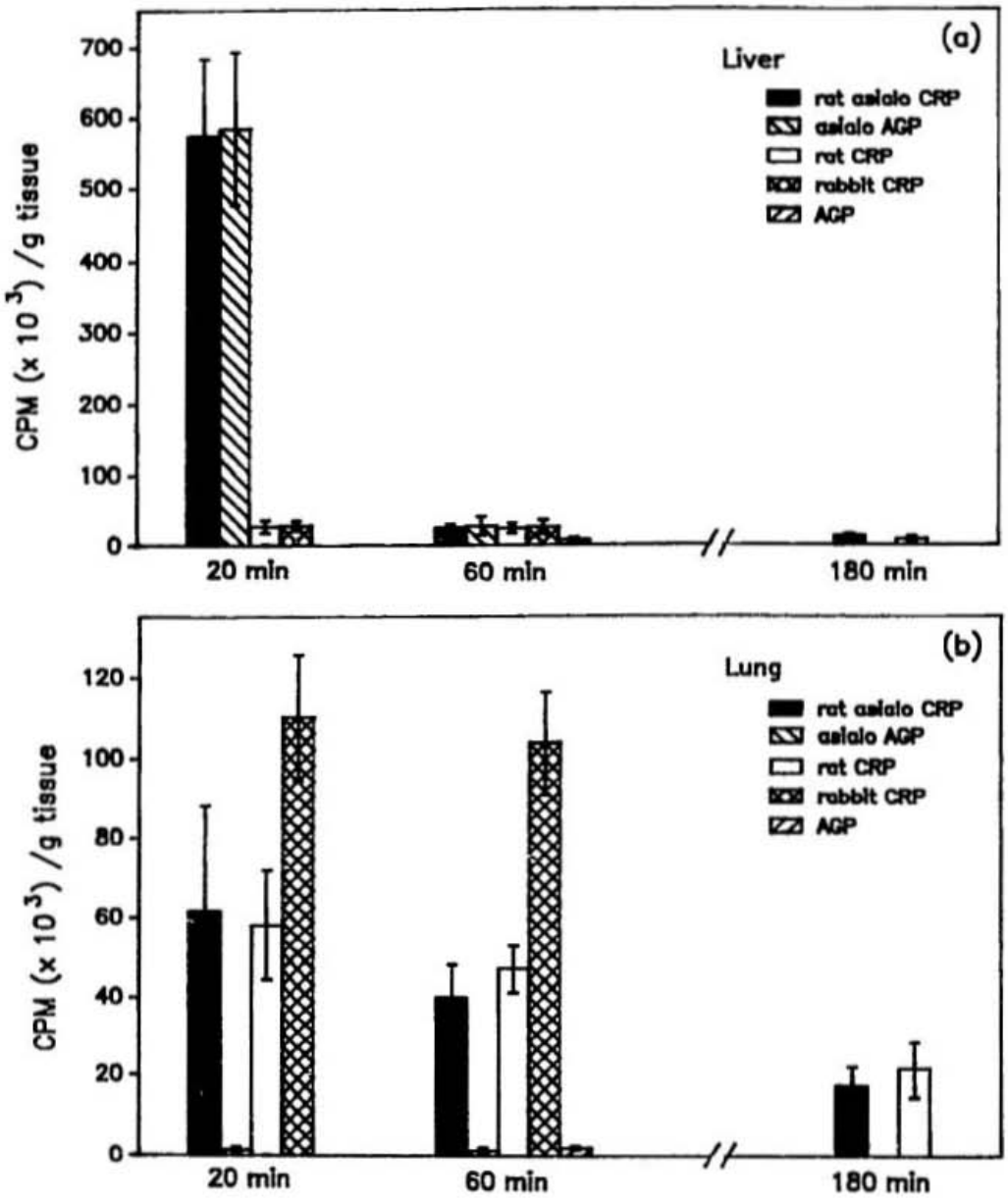


Fig. 22. **Tissue distribution of radioactivity 20 min after the injection of ^{125}I-labeled rat asialo CRP.** Twenty min after the injection of ^{125}I-labeled rat asialo CRP (12 μg, 9 x 10^6 CPM), radioactivity in different tissues was determined as described in chapter 2, pp. 47-48. Results shown were obtained from two separate experiments. Error bars in the graph represent the confidence limits with 95% probability.

Conditions	rat asialo CRP	rat CRP	rabbit CRP	asialo AGP
CPM injected ($x10^6$)	9	9	9	9
% cleared from circulation after 20 min	92 ± 3.1%	6 ± 2.7%	5 ± 2.3%	90 ± 4.2%
CPM recovered in liver ($x10^6$)	7.1 ± 1.3	0.3 ± 0.1	0.3 ± 0.1	6.9 ± 1.2
Recovery of cleared radioactivity in liver	86 ± 14%	60 ± 16%	65 ± 22%	85 ± 15%
CPM recovered in lung ($x10^4$)	6.9 ± 2.7	6.4 ± 1.9	14 ± 2.4	0.5 ± 0.1
Recovery of cleared radioactivity in lung	0.8 ± 0.3%	12 ± 3.6%	30 ± 5.1%	0.05 ± 0.01%

Table 4. **Recovery of radioactivity in liver and lungs.** Twenty min after the injection of ^{125}I-labeled rat asialo CRP, rat CRP, rabbit CRP and asialo AGP, radioactivity in liver and lungs was determined as described in chapter 2, pp. 47-48. The values in this table are the means and their confidence limits with 95% possibility.

Fig. 23 (a & b). **Time-dependent distribution of radioactivity in liver and lungs.** At 20 min, 60 min or 180 min after the injection of ^{125}I-labeled rat asialo CRP, rat and rabbit CRP, AGP or asialo AGP (9×10^6 CPM), radioactivity in liver (Fig. 23a) and lungs (Fig. 23b) was determined as described in chapter 2, pp. 47-48. Results shown were obtained from two separate experiments (for AGP, the results were obtained from one experiment). Error bars in the graph represent the confidence limits with 95% probability.


(a)
Liver
rat asialo CRP
asialo AGP
rat CRP
rabbit CRP
AGP
CPM (x 10^3) /g tissue
700
600
500
400
300
200
100
0
20 min
60 min
180 min
(b)
Lung
rat asialo CRP
asialo AGP
rat CRP
rabbit CRP
AGP
CPM (x 10^3) /g tissue
120
100
80
60
40
20
0
20 min
60 min
180 min

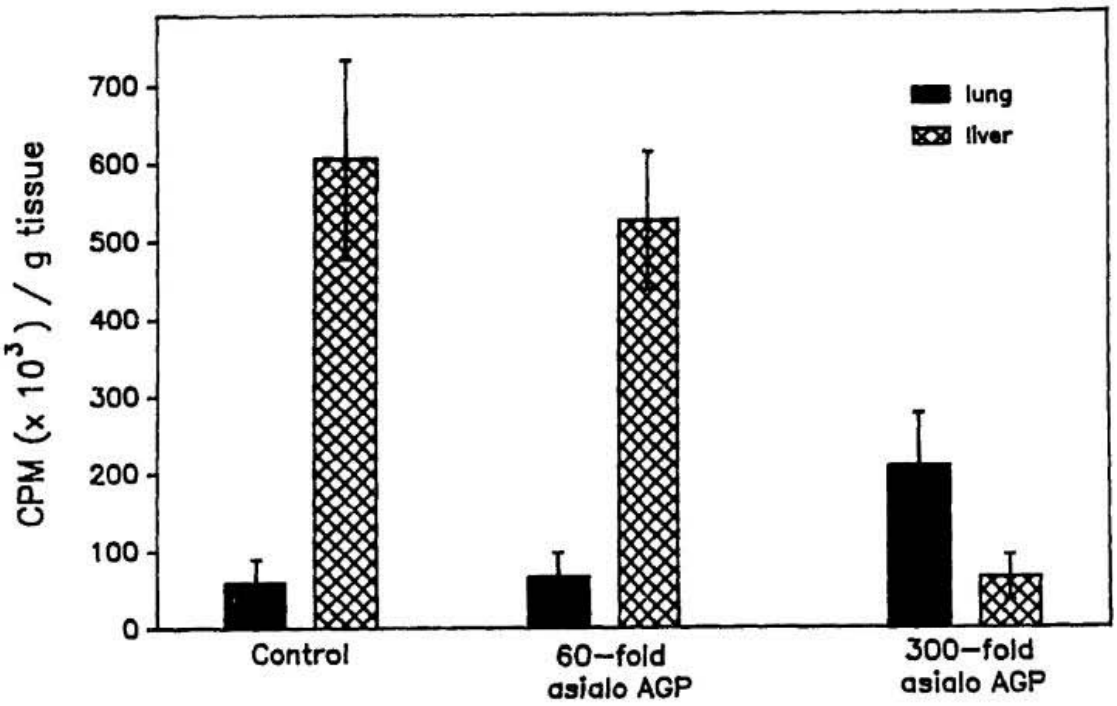


Fig. 24. **Effect of asialo AGP on the distribution of radioactivity associated with ^{125}I-labeled rat asialo CRP in liver and lungs.** Twenty min after the injection of 15 μg of ^{125}I-labeled rat asialo CRP and either 300 μg (60-fold excess) or 1.5 mg (300-fold excess) of asialo AGP, radioactivity in liver and lungs was determined as described in chapter 2, pp. 47-48. Control refers to the distribution of radioactivity 20 min after the injection of ^{125}I-labeled rat asialo CRP in the absence of asialo AGP. Results shown were obtained from two separate experiments. Error bars in the graph represent the confidence limits with 95% probability.

rat asialo CRP showed an increase in lungs (Fig. 24).

D. HPLC ANALYSIS OF THE RADIOACTIVITY IN LIVER AND LUNG HOMOGENATES

In order to determine if there was any degradation of the injected ^{125}I-labeled rat asialo CRP in liver and lungs, the radioactivity bound to the liver and lungs was released by EDTA treatment and analyzed by HPLC using a gel-filtration column. Typically, EDTA treatment resulted in the release of >50% of the radioactivity from the tissue. When the supernatant obtained after EDTA treatment of the liver and lung homogenates was applied to the column, about 20-30% of the radioactivity eluted at a position which corresponded to intact rat asialo CRP. A small peak (about 10% of the total area) appeared at an earlier time (7 min), which may represent aggregated rat asialo CRP. The remaining 60-70% eluted at 15-18 min, which could be due to some low molecular weight fragments (Fig. 25 a & b). This indicates that the majority of the rat asialo CRP was degraded in liver and lungs, Assuming that EDTA treatment or homogenization of the tissues did not contributed to the degradation of rat asialo CRP.

E. CLEARANCE EXPERIMENTS USING PERFUSED LIVER

The clearance of rat CRP and rat asialo CRP was further studied using perfused liver. The results obtained in this system were comparable to that observed in whole animals.

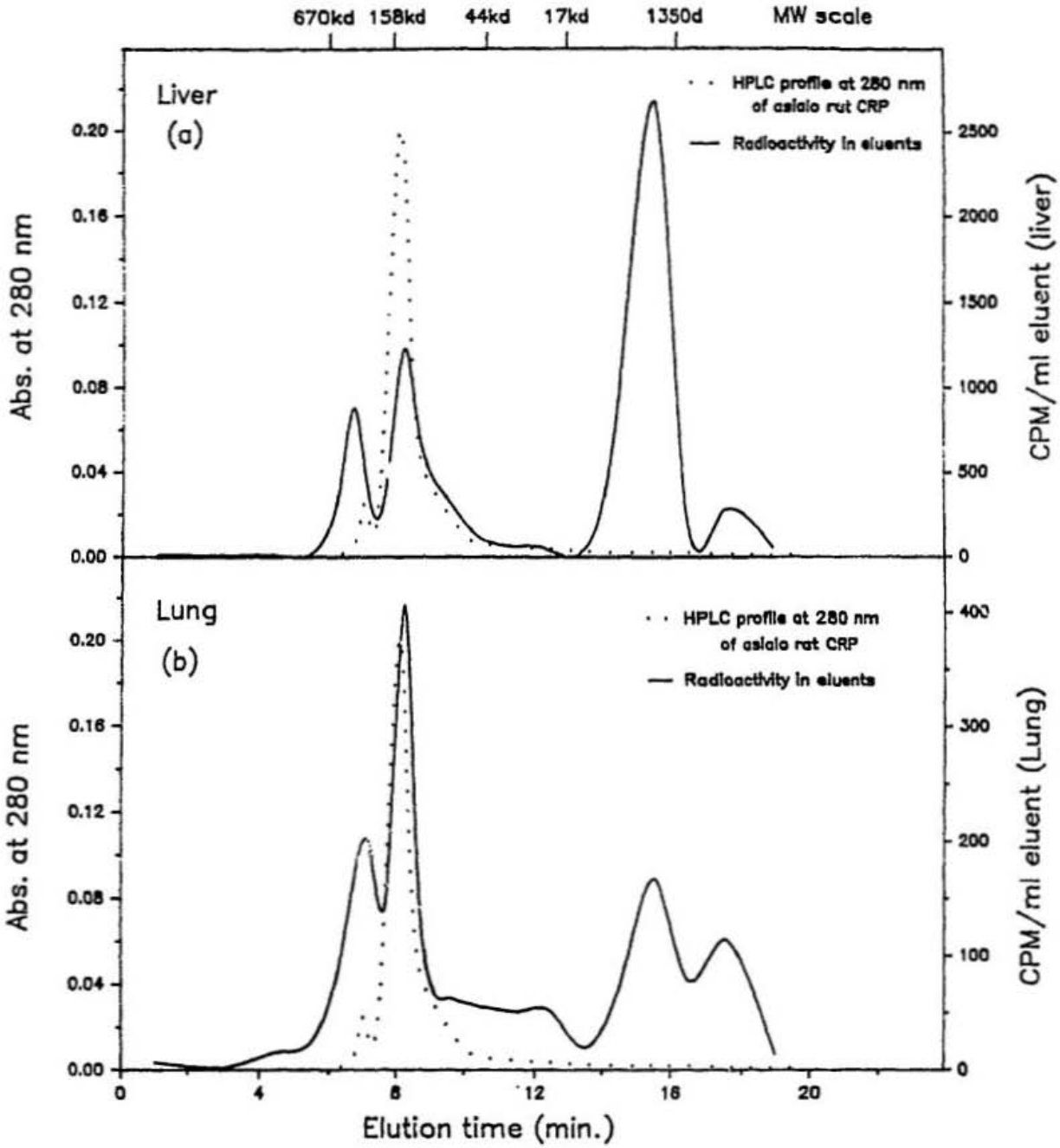


Fig. 25 (a and b). **HPLC profiles of the radioactivity released from liver and lung homogenates.** Homogenates of liver (Fig. 25a) and lungs (Fig. 25b) obtained 20 min after the injection of 30 μg of labeled rat asialo CRP were treated with 8 mM EDTA. The released radioactivity was analyzed by HPLC as described in chapter 2, pp. 46-47.

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^{125}I-labeled rat asialo CRP and asialo AGP were cleared by the perfused liver, while ^{125}I-labeled rat CRP remained in the perfusion medium during the procedure which lasted for 60 min (Fig. 26).

The hepatic asialoglycoprotein receptor is known to be a glycoprotein whose terminal sialic acids are involved in the clearance of asialoglycoproteins (Ashwell and Harford, 1982). After the perfused liver had been treated with neuraminidase, the clearance of rat asialo CRP was completely inhibited (Fig. 27).

When GalNAc, a strong inhibitor of the binding of asialoglycoprotein to the hepatic asialoglycoprotein receptor, was included in the perfusion medium, the clearance of ^{125}I-labeled rat asialo CRP by the perfused liver was inhibited (Fig. 27).

To examine if the phosphorylcholine binding domain of rat asialo CRP had any effect on its clearance, phosphorylcholine (1 mM) was included in the perfusion medium. No inhibition of the clearance of ^{125}I-labeled rat asialo CRP was observed (Fig. 27). One mM phosphorylcholine has been shown previously to be more than sufficient to inhibit the binding of rat asialo CRP to Sepharose-phosphorylcholine affinity column (Nagpurkar and Mookerjea, 1981).

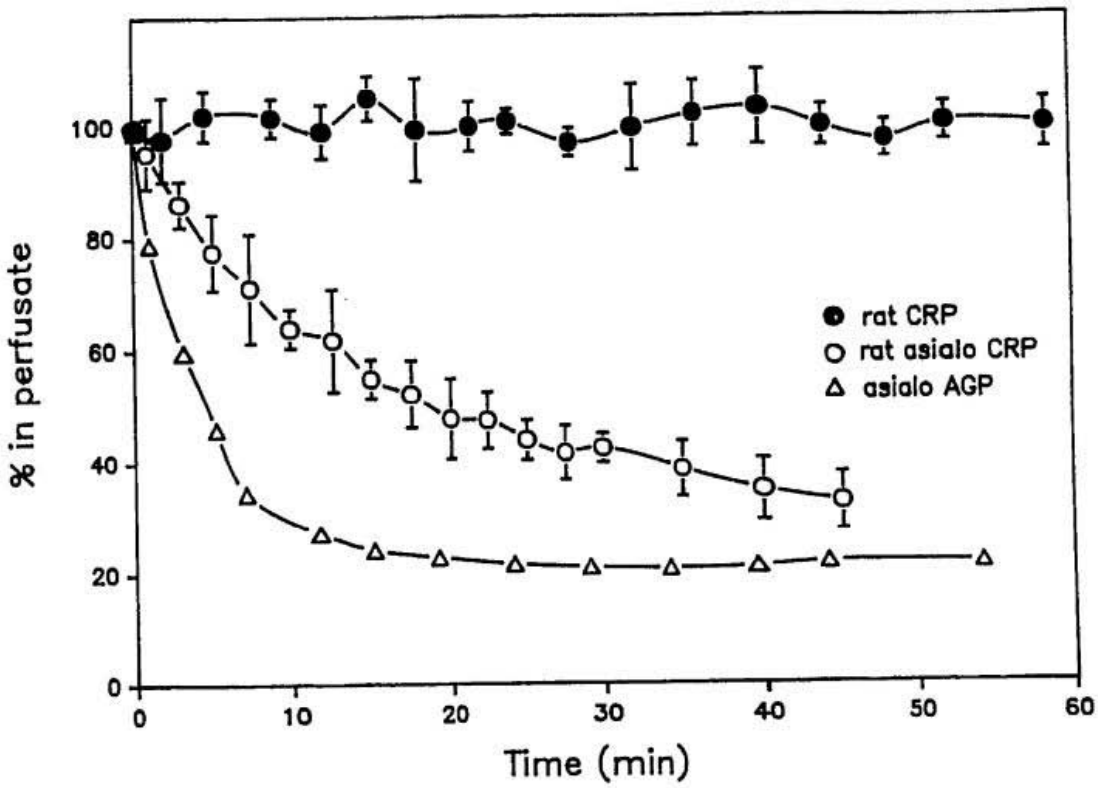

Fig. 26. **Clearance of rat asialo CRP, rat CRP and asialo AGP by perfused liver.** Rat liver was perfused through the portal vein with oxygenated Hanks' buffer at 37°C for 5 min as described in chapter 2, pp. 49-50. The perfusion solution was then changed to 70 ml of Waymouth's medium containing 15 μg of ^{125}I-labeled protein samples and recirculated. The liver was perfused for 60 min at a flow rate of 30 ml/min. At the specified time intervals, a sample of the perfusate was taken and the radioactivity in the perfusate determined as described in chapter 2, pp. 49-50. The amount of radioactivity in the perfusate at different times has been expressed as the percentage of that at 0 time. In the case of rat CRP and rat asialo CRP, each point in the graph represents the mean ± 2 SD of two values from two separate experiments.

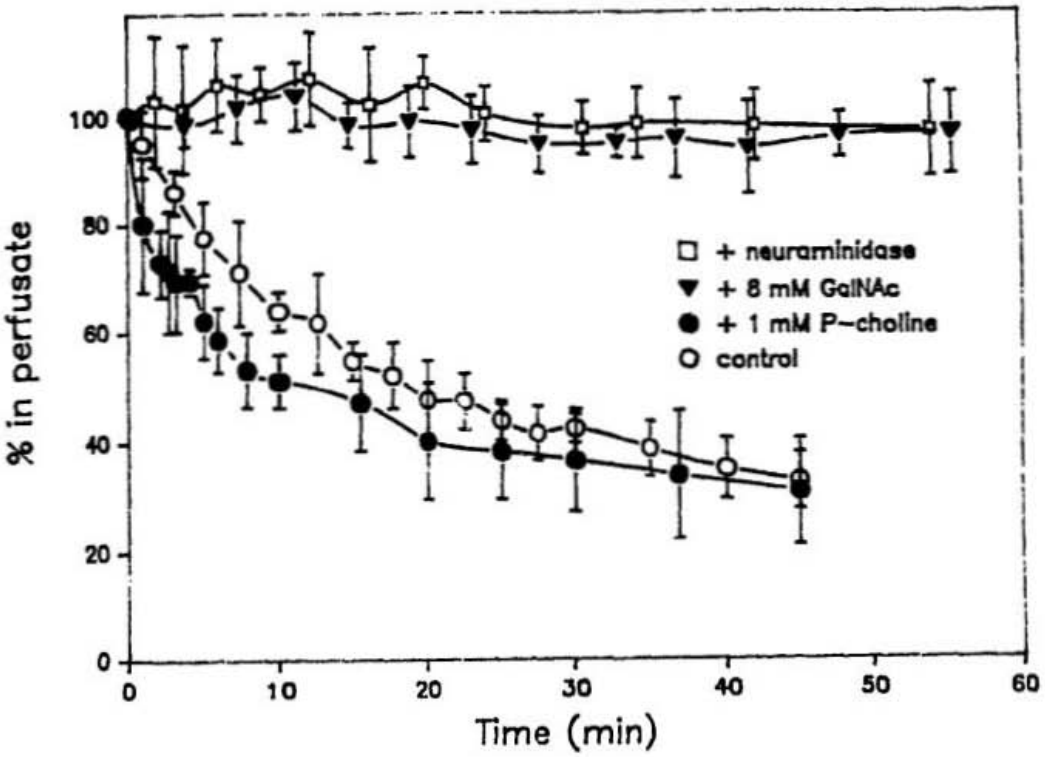

Fig. 27. Effect of GalNAc, phosphorylcholine and neuraminidase on the clearance of rat asialo CRP by perfused liver. In experiments to study the effect of GalNAc and phosphorylcholine on the clearance of rat asialo CRP, a final concentration of 8 mM GalNAc or 1 mM phosphorylcholine was included in the perfusion medium; to study the effect of neuraminidase on the clearance of rat asialo CRP, the liver was first perfused with Waymouth's medium containing 0.5 units of neuraminidase for 30 min, followed by perfusing with 30 ml of fresh Waymouth's medium to remove the neuraminidase. The liver was then perfused with 70 ml of fresh Waymouth's medium containing 15 μg of labeled rat asialo CRP. Other experimental details and calculations are the same as described in the legend for Fig. 26. Control refers to the clearance of rat asialo CRP by the perfused liver in the absence of GalNAc, phosphorylcholine or neuraminidase. Each point in the graph is the mean ± 2 SD of two separate experiments.

Chapter 4 DISCUSSION

The objective of this study was to examine the clearance mechanism of CRP. Based on Ashwell's observations, it is reasonable to speculate that glycosylated CRP, after desialylation, would be taken up by the hepatocytes through the hepatic asialoglycoprotein receptor. This study demonstrated the clearance of glycosylated CRP through Ashwell's pathway and examined the role of the phosphoryl-choline binding domain of both glycosylated and non-glycosylated CRP in their clearance.

A. BINDING OF RAT ASIALO CRP, RAT CRP AND RABBIT CRP TO ISOLATED HEPATOCYTES

The binding of asialo AGP to isolated hepatocytes was saturable, and was inhibited by GalNAc (Fig. 12, insert and Fig. 14a). Based on the binding of asialo AGP to the hepatocytes, the number of the hepatic asialoglycoprotein receptors on the surface of the hepatocytes was found to be comparable with published results (Table 3). Therefore, it was concluded that the asialoglycoprotein receptors on these hepatocytes were functional.

However, it was observed that the binding of both rat asialo CRP and rat CRP to the hepatocytes was much higher than that of asialo AGP (Fig. 12). The amount of rat asialo CRP and rat CRP bound to the hepatocytes was 35 and 15

times, respectively, more than that of asialo AGP (Fig. 12). Although the binding of rat asialo CRP was about twice that of rat CRP (Fig. 12), the increased binding of rat asialo CRP could not be attributed to the binding of this protein to the hepatic asialoglycoprotein receptor, since GalNAc did not reduce the binding of rat asialo CRP to the hepatocytes (Fig. 14a). Consequently, it was reasonable to believe that rat asialo CRP did not bind to the hepatic asialo-glycoprotein receptor, and glycosylated CRP might be an exception to Ashwell's pathway.

The increased binding of rat asialo CRP to the hepatocytes, over that of rat CRP, can be attributed to the enhanced accessibility of the asialo protein to the binding site, as a consequence of the reduced net negative charge after desialylation. A similar observation was made during the binding of human CRP to red blood cells. The binding of human CRP to desialylated red blood cells was about twice over that to normal red blood cells (Volanakis, 1982a).

In view of the common phosphorylcholine binding property of CRP, the effect of phosphorylcholine on the binding of rat asialo CRP, rat CRP and rabbit CRP to hepatocyte was examined. Phosphorylcholine inhibited the binding of these CRP to the hepatocytes, but had no effect on the binding of asialo AGP (Fig. 14b). It was therefore concluded that these CRP bound to a common binding site on the hepatocytes through their phosphorylcholine binding

domain. Additional support of this concept was provided by the competitive inhibition of the binding of labeled rat asialo CRP to the hepatocytes by rat CRP. In the presence of rat CRP, the binding of labeled rat asialo CRP to the hepatocytes was reduced by about 60% (Fig. 17).

The above results, however, could not explain why asialo CRP had no interaction with the hepatic asialoglycoprotein receptor. One reason could be that in the presence of large amounts of phosphorylcholine-mediated binding of rat asialo CRP to the hepatocytes, the binding of rat asialo CRP to the asialoglycoprotein receptor could not be detected. This possibility was examined by competitive inhibition studies using rat asialo CRP and asialo AGP. Hepatocytes were pre-incubated with rat asialo CRP before the addition of labeled asialo AGP. The results showed that the binding of labeled asialo AGP was reduced by as much as 80% by rat asialo CRP pre-bound to the hepatocytes (Fig. 15). In contrast, pre-incubating the hepatocytes with rat CRP had no effect on the binding of labeled asialo AGP (Fig. 17). These results demonstrate that the exposed galactose residues of rat asialo CRP are responsible for the inhibition of the binding of asialo AGP to the hepatocytes, and that rat asialo CRP binds to the asialoglycoprotein receptor on the hepatocytes. It was also shown that when hepatocytes which had been pre-incubated with rat asialo CRP

were treated with GalNAc, the binding of labeled asialo AGP to the hepatic asialoglycoprotein receptor was restored (Fig. 16). This indicates that rat asialo CRP bound to the hepatic asialoglycoprotein receptor can be released by GalNAc, and that the binding of rat asialo CRP to this receptor is reversible.

In order to determine if the large amounts of binding of rat asialo CRP through its phosphorylcholine binding domain could also be responsible for the competitive inhibition of asialo AGP binding, hepatocytes were pre-incubated with rat asialo CRP and treated with phosphoryl-choline. The binding of labeled asialo AGP to the hepatocytes was virtually unchanged with this treatment (Fig. 16). On the other hand, phosphorylcholine has been shown to reduce the binding of rat asialo CRP by as much as 75% (Fig. 14b). These results indicate that the binding of rat asialo CRP through its phosphorylcholine binding domain is distinct from its binding to the hepatic asialo-glycoprotein receptor.

A rational explanation for the failure to directly observe the binding of rat asialo CRP to the hepatic asialoglycoprotein receptor is that the phosphorylcholine involved binding sites on the isolated hepatocytes far out-number the hepatic asialoglycoprotein receptors. Under the experimental conditions used, it is not possible to detect

the much smaller amount of binding of rat asialo CRP to the hepatic asialoglycoprotein receptor.

To explain the observed large amounts of binding of CRP to the isolated hepatocytes, binding assays using isolated hepatocytes with different viability were carried out. As the viability of the hepatocytes decreased, the binding of rat asialo CRP to the hepatocytes increased (Fig. 18). It is assumed here that there is a correlation between reduced cell viability and greater disruption of the cell surface. It can be speculated that disruption of the hepatocyte surface generates binding sites containing the phosphoryl-choline ligand. Several examples are available in the literature in support of this concept. i) Human CRP has been shown to bind more avidly to artificial bilayer membranes that have been structurally altered (Volanakis and Wirtz, 1979; Narkates and Volanakis, 1982); ii) Previous work in this laboratory on the binding of rat CRP to liposomes has shown that the binding of rat CRP to the liposomes increased when lysolecithin was incorporated into the phosphatidyl-choline containing liposomes (Nagpurkar et al., 1983); iii) The binding of human CRP to red blood cells was shown to be enhanced when the red cells were treated with phospholipase A_2 or when ghost cells were used (Narkates and Volanakis, 1982); and iv) Rabbit CRP has been shown to deposit in damaged tissue during inflammation (Kushner and Kaplan, 1961; Gitlin et al., 1978; Volanakis and Narkates, 1981;

DuClos and Mold, 1981).

B. IN VIVO CLEARANCE OF RAT ASIALO CRP, RAT AND RABBIT CRP

The clearance of rat asialo CRP, rat and rabbit CRP was examined *in vivo* and using perfused liver.

While rat and rabbit CRP remained in circulation for hours, more than 50% of the labeled rat asialo CRP was removed from circulation within 4 min (Fig. 19). The clearance of labeled rat asialo CRP was inhibited by asialo AGP and asialo-fetuin (Fig. 20 & 21), but was not affected by AGP (Fig. 21). These results suggest that rat asialo CRP shares a common clearance pathway with other asialo-glycoproteins.

Using a liver perfusion system, it was shown that the clearance of labeled rat asialo CRP by the perfused liver was inhibited by GalNAc or by pre-treatment of the liver with neuraminidase (Fig. 27). These results provide conclusive evidence that rat asialo CRP is cleared *in vivo* by the hepatic asialoglycoprotein receptor (Ashwell's pathway).

It has been demonstrated that the binding affinity of asialoglycoproteins for the asialoglycoprotein receptor depends on the carbohydrate antennary structure of the glycoproteins. The binding affinity to the hepatic asialo-glycoprotein receptor decreases in the order of tetra-> tri->> bi-> monoantennary oligosaccharide chains (Lee et

al., 1983; Bezouska et al., 1985). The reduced binding affinity results in slower clearance of the glycoproteins. The carbohydrate structure of rat CRP has been shown to be biantennary (Sambasivam et al., 1990), while that of AGP is mainly triantennary. This difference in carbohydrate structure may explain the slower clearance rate of rat asialo CRP compared to that of asialo AGP.

In vitro binding assays showed large amounts of binding of rat asialo CRP, rat and rabbit CRP to the hepatocytes through their phosphorylcholine binding domain (Fig. 12 & 14b). In the liver perfusion system, phosphorylcholine was shown to have no effect on the clearance of rat asialo CRP by the perfused liver (Fig. 27). This indicates that the phosphorylcholine binding domain of rat asialo CRP does not affect its clearance through Ashwell's pathway.

As a comparison to the fast clearance of rat asialo CRP from the circulation, the half-life of rat CRP was determined using five rats (Fig. 19, insert). Each point in the graph represents the data obtained from one rat. Even so, the half-life of rat CRP obtained in this study is comparable with previously reported values (Baltz et.al. 1985).

After the clearance of labeled rat asialo CRP, most of the radioactive label was recovered in the liver (Fig. 22 and table 4). However, some radioactive label was also found in the lungs (Table 4). Besides rat asialo CRP, rat CRP and

rabbit CRP were found to be taken up by the lungs as well (Fig. 23 and Table 4). In contrast, no radioactive label associated with AGP and asialo AGP was recovered in the lungs. This suggests that there may be a unique relationship between CRP and lungs.

A possible reason for the observed accumulation of CRP in the lungs is that ether treatment might cause damage to the lungs, and thus lead to the binding of CRP to the damaged lung tissue. To clarify this point, some experiments on the clearance of rat asialo CRP were carried out in rats without using ether anaesthesia. The rats were restrained at the time of sample injection. The results obtained under these conditions were identical to those obtained with ether treatment. Therefore, the uptake of CRP by the lungs appears to be a physiological event in rats.

When the clearance of labeled rat asialo CRP was competed by increasing amounts of asialo AGP (Fig. 20), the recovery of radioactivity decreased in the liver (Fig. 24). At the same time, there was a corresponding increase of radioactivity associated with rat asialo CRP in the lungs (Fig. 24). The apparent connection between the clearance of rat asialo CRP in liver and in lungs is presently not clear.

HPLC profiles of the radioactivity associated with liver and lungs showed that about 60-70% of the rat asialo CRP was degraded (Fig. 25). Macrophages are abundant in lungs, and it has been demonstrated that CRP bind to

macrophages (Kindmark, 1972; Kempka et al., 1990). It is therefore reasonable to speculate that CRP could be taken up and degraded by the macrophages in the lungs. The physiological implications of this possibility are under investigation in our laboratory.

A general scheme of the clearance of glycosylated and non-glycosylated CRP by the liver and the lungs is shown in Fig. 28. Pathway one (denoted by a cycled 1) is proposed to be the common clearance rout for all CRP, either glycosylated or non-glycosylated CRP. Pathway two (denoted by a cycled 2), on the other hand, can only be utilized by glycosylated CRP.

C. CONCLUSION AND FUTURE DIRECTIONS OF RESEARCH

In conclusion, rat asialo CRP was shown to bind to the hepatic asialoglycoprotein receptor and be cleared *in vivo* mainly through this receptor. The binding of rat asialo CRP, rat CRP and rabbit CRP to isolated hepatocyte, however, was predominantly mediated through their phosphorylcholine binding domain to binding sites generated as a consequence of a disruption of the surface of the isolated hepatocytes. Despite the large amount of binding of rat asialo CRP to the hepatocytes through the highly conserved phosphorylcholine binding domain, the clearance of rat asialo CRP via the hepatic asialoglycoprotein receptor was not affected. Furthermore, results from this study indicate that there

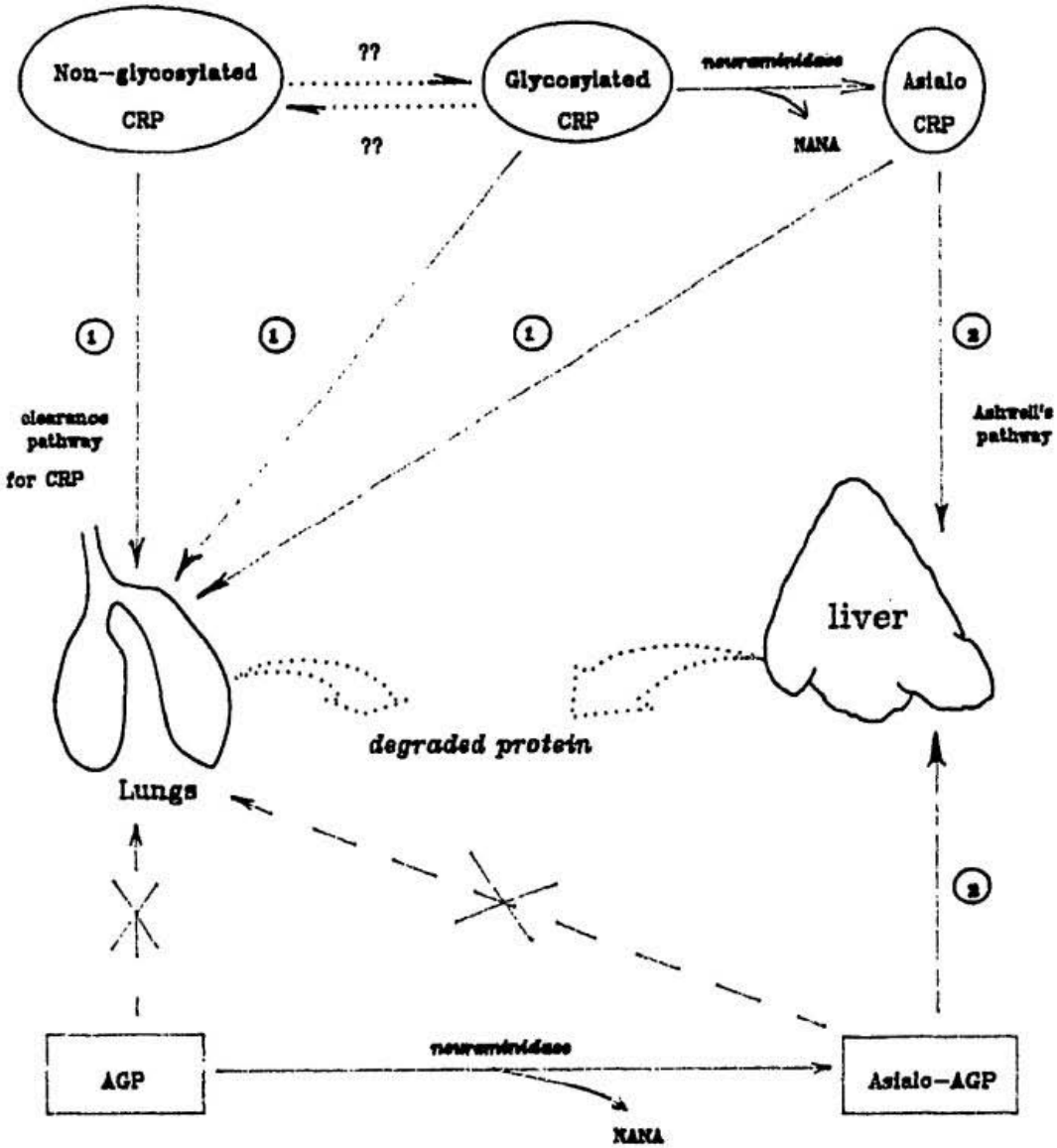

Fig. 28. Clearance of glycosylated and non-glycosylated CRP in liver and lungs

could be an as yet to be identified pathway for the clearance of CRP in the lungs.

Hepatocytes in diseased states have been shown to have altered membrane structure (Bikhazi et al., 1989). Elevation of CRP level in serum has been observed in many diseases associated with inflammation or tissue destruction, particularly in rheumatoid disease, acute infectious processes, postmyocardial infarct, advanced and widespread malignancy, and acute and chronic infections (Anderson and McCarty, 1950; Stollerman et al., 1953; Koj, 1974; Kushner et al., 1978b). It has been suggested that the binding of human CRP to the damaged membranes triggers a cascade of immuno- related reactions leading ultimately to the repair of the damaged tissue (Kushner et al., 1981; Volanakis and Narkates, 1981; Robey et al., 1983). In this context, it would be meaningful to determine if there is a markedly increased binding of CRP to diseased cells and to characterize the binding site for CRP on cell membranes. This may contribute to a better understanding of the physiological functions of CRP.

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