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The role of phosphate groups in the interaction of human C-reactive protein with galactan polysaccharides

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SUMMARY

Human C-reactive protein (CRP) shows binding specificities for phosphate monoesters, polycations and for several other biological macromolecules lacking these ligands. We report here that the formerly observed interaction of CRP with snail galactans, as exemplified by *Helix pomatia* galactan, is not due to a lectin-like carbohydrate-binding reactivity, but, instead that CRP obviously binds to phosphate groups that are minor constituents of these polysaccharides. Structural analysis of the galactan revealed that the phosphate groups are attached by a, as yet unidentified, linkage group to the carbohydrate backbone. Thus, the anti-galactan reactivity of CRP can be attributed to the protein's classical anti-phosphate/anti-phosphorylcholine specificity.

INTRODUCTION

C-reactive protein (CRP), the classical acute phase reactant of human serum (Tillet & Francis, 1930), undergoes reactions of precipitation (Tillet & Francis, 1930), agglutination (Gál & Miltényi, 1955) and activation of complement by the classical reaction pathway (Kaplan & Volanakis, 1974). In an aggregated or ligand-complexed form, the protein binds to human low-density and very low-density lipoproteins (De Beer *et al.*, 1982). Aggregated or complexed CRP also interacts with human blood cells: CRP opsonizes particles and cells for phagocytosis by monocytes and granulocytes (Mold *et al.*, 1982), it binds to lymphocytes bearing the Fc receptor and alters some of their functions (Vetter *et al.*, 1983), and promotes platelet activation and aggregation (Fiedel, Simpson & Gewurz, 1982).

All these biological functions are supposed to be a direct consquence of the molecule's binding reactivities. The calcium ion-dependent binding specificity for phosphorylcholine and other phosphate monoesters (Gotschlich & Edelman, 1967; Volanakis & Kaplan, 1971) and a second binding reactivity for polycations (DiCamelli *et al.*, 1980) are well characterized. Furthermore, CRP binds to certain polysaccharides (Heidelberger, Gotschlich & Higginbotham, 1972; Pepys, Dash & Ashley, 1977; Uhlenbruck *et al.*, 1979), to fibronectin (Salonen *et al.*, 1984), and to chromatin (Robey *et al.*, 1984). However, the actual CRP ligands in these macromolecules have not been identified.

Several reports describe interactions *in vitro* of CRP with D-galactose-containing polysaccharides, including agarose (Pepys *et al.*, 1977; Volanakis & Narkates, 1982) and depyruvylated *Streptococcus pneumoniae* type 4 polysaccharide (Heidelberger *et al.*, 1972). We previously reported that CRP and also human serum amyloid P protein and the complement subcomponent C1q precipitate *in vitro* a number of snail galactans: polysaccharides which are found in the albumin glands and egg masses of certain snail species (Uhlenbruck *et al.*, 1979, 1981). However, it has not been demonstrated definitely that CRP really has a lectin-like binding specificity for galactose or any other sugar. In view of the growing acceptance that carbohydrate-binding proteins may play fundamental roles in biological recognition processes, including non-specific immune response (for a review see Barondes, 1981), the unequivocal demonstration that CRP is indeed a lectin may cast some light on the unknown physiological function of this serum protein. Therefore, the present study was designed to characterize further these CRP:galactan interactions in order to identify the actual ligand(s) in these polymers.

Abbreviations: CRP, C-reactive protein; HF, hydrofluoric acid; HMDS, hexamethyldisilizane; HPG, *Helix pomatia* galactan; MSTFA, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; ONPG, *o*-nitrophenyl β-*D*-galactopyranoside; PBS, phosphate-buffered saline; PC, phosphorylcholine; TBS-Ca^{2+}, Tris buffered saline containing calcium chloride; TFA, trifluoroacetic acid; TMCS, trimethylchlorosilane; X-P, phosphate containing linkage group released from HPG by the action of 48% HF.

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MATERIALS AND METHODS

Purified human CRP was a generous gift from Behring-Werke (Marburg, FRG). The preparation gave a single polypeptide band at MW 21,000 in sodium dodecyl sulphate polyacrylamide gel electrophoresis. *Helix pomatia* snails were collected near Kerpen, West Germany, in early June. *Helix aspersa* snails were collected in the Vaucluse Department (France). The latter galactan was prepared from the albumin glands by extraction

with saline. *Tridacna maxima* clams were collected from the North Fisheries Station, Queensland Fisheries Service (Australia) and shipped per air mail in a frozen state. The clam haemolymph lectin was purified by affinity chromatography with acid-activated Sepharose 4B (Pharmacia, Freiburg, FRG) as described previously (Baldo *et al.*, 1978). Pneumococcal C polysaccharide, prepared from the cell wall of a Cs capsulated strain of *Streptococcus pneumoniae*, was kindly supplied by Dr G. Schiffman (Brooklyn, NY). The phosphorylcholine-binding IgA mouse myeloma protein TEPC 15 was kindly supplied by Prof. K. Rajewsky (University of Cologne). *Ricinus communis* agglutinin (RCA-1) was from Boehringer (Mannheim, FRG). Alkaline phosphatases from bovine intestine (1 u/mg) and from *Escherichia coli* (20–30 u/mg) were from Sigma (München, FRG). *o*-Nitrophenyl β-D-galactopyranoside (ONPG), larch arabinogalactan and all the sugars and phosphate esters used for inhibition studies were obtained from Serva (Heidelberg, FRG). Silicone OV-225, 3% on Chromosorb W, silicone SE-52, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), hexamethyldisilazane (HDMS), trimethylchlorosilane (TMCS) as Silyl-21 (HMDS/TMCS, 2:1) were purchased from Macherey & Nagel (Dueren, FRG). Aqueous hydrogen fluoride (48%) was obtained from Riedel-de-Haen (Seelze, FRG).

Purification of Helix pomatia *galactan*
A crude saline extract of snail albumin glands was delipidified with chloroform:methanol (2:1, v/v) and then further purified by affinity chromatography with immobilized *Tridacna maxima* lectin (Tridacnin) as described by Gleeson, Jermyn & Clarke (1979). Contaminating proteins were subsequently separated by gel filtration on Biogel A-5 m (Bio-Rad, München, FRG) in phosphate-buffered saline (PBS). After treatment of this product with 20% potassium hydroxide at 100° for 90 min, a galactan preparation free from protein was obtained.

Ultraviolet difference spectroscopy
Difference spectra were recorded on a Beckman 24 double-beam spectrophotometer at ambient temperature using matched double-compartment cuvettes as described by Bessler, Shafner & Goldstein (1973). The tests were performed with 1 mg/ml CRP or purified Tridacnin in 0·1 M Tris-HCl buffer, pH 7·2, containing 0·15 M NaCl and 0·01 $CaCl_2$ (TBS-Ca^{2+}) with 250 μM ONPG as the perturbant.

Quantitative precipitation assay and precipitation inhibition assay
Microprecipitin tests were performed as described by Kabat & Mayer (1961) with following modifications: 25 μg CRP were incubated with increasing amounts of *Helix pomatia* galactan (HPG) or larch arabinogalactan (0–150 μg) in a total valume of 50 μg TBS-Ca^{2+} buffer for 1 hr at 37° and overnight at 4°. The protein contents of the washed precipitates (dissolved in 100 μl distilled water) were determined according to Lowry *et al.* (1951). The inhibitory effects of low MW substances were tested by preincubating 25 μg CRP with increasing amounts of inhibitor substances (0–10 mM) for 15 min at room temperature before adding the amount of HPG required for maximal precipitation in the absence of inhibitor (total volume 50 μl).

Treatment of HPG with alkaline phosphatases
Five mg HPG dissolved in 1 ml 0·1 M carbonate buffer, pH 9·8, were incubated with 0·1 u of alkaline phosphatase from bovine intestine or from *Escherichia coli* for 21 hr at 25°. The reactions were stopped by heating in a boiling water bath for 5 min.

Treatment of polysaccharides with hydrofluoric acid
For dephosphorylation, samples of different polysaccharides were dissolved to 5% in 48% hydrofluororic acid (HF) and incubated at 4° for 1–24 hr (Mort & Lamport, 1977). The reactions were stopped by quick diluting of the samples with cold distilled water and subsequent lyophilization.

Gel chromatography
Five mg HPG were reacted with HF for 4 hr as described in the previous section. After removal of HF by lyophilization, the sample was dissolved in distilled water and chromatographed on Sephadex G-25 (Pharmacia) (26 × 1·1 cm) in distilled water with a flow rate of 5 ml/hr.

Agarose gel diffusion test
Double diffusion tests were performed in 1% gels of agarose (Serva, Heidelberg, FRG) in TBS-Ca^{2+} buffer (Ouchterlony, 1948).

Gas chromatography
For analyses, samples of HPG were hydrolysed in 0·7 N HCl (100°, 4 hr) and then lyophilized. Monosaccharide and monosaccharide phosphate analyses were performed by gas liquid chromatography after trimethylsilylation with MSTFA/trifluoroacetic acid (10:1, v/v) on silicone OV-225 (Chaplin, 1982) and with HMDS/TMCS (2:1) on silicone SE-52 (Leblanc & Ball, 1978), respectively.

Other methods
Total protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard, total carbohydrate was estimated according to Dubois *et al.* (1956) with D-galactose as a standard, inorganic and total phosphate were determined according to Ames (1966) with potassium dihydrogen phosphate as a standard, choline was estimated according to Barak & Tuma (1981).

RESULTS

Ultraviolet difference spectroscopy

In an attempt to determine a binding specificity of CRP for galactose residues, ultraviolet difference spectra were recorded using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as the carbohydrate ligand. Figure 1 shows that no difference could be detected between the UV absorbance spectra of CRP in the 240–350 nm region without or in the presence of 250 μM ONPG. In contrast, the addition of the same amount of ONPG to a solution of Tridacnin, the D-galactose specific haemolymph lectin from *Tridacna maxima* clams, yielded a characteristic UV difference spectrum with a negative difference maximum at 270 nm (Fig. 1).

Precipitin reactions of CRP with polysaccharides

CRP reacted with purified *Helix pomatia* galactan in agarose gels containing TBS-Ca^{2+} buffer forming sharp precipitation lines (Fig. 2). However, there was no reaction when performing

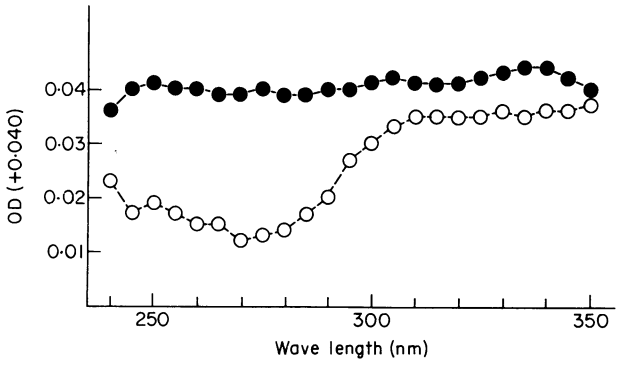

Figure 1. Untraviolet difference spectra of (●——●) CRP (1 mg/ml) and (○- - -○) Tridacnin from *Tridacna maxima* (1 mg/ml) produced by 250 μM *o*-nitrophenyl β-D-galactopyranoside. Buffer: 0·01 M TBS-Ca^{2+}.

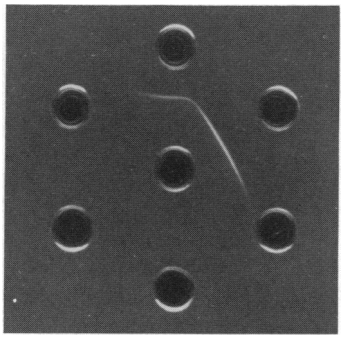

Figure 2. Double diffusion test in agarose gel. Substances from 12 o'clock (=1) are clockwise: 1 = *Helix pomatia* galactan, 2 = *Streptococcus pneumoniae* C polysaccharide, centre well = CRP 1% in 3% citrate. The polysaccharides were tested at 1% in 0·01 M TBS-Ca^{2+}.

the test in PBS buffer or when replacing calcium chloride by 10 mM ethylenediamine tetraacetate (EDTA). When testing CRP and the galactan together with *Streptococcus pneumoniae* C polysaccharide, completely fusing precipitation lines were obtained, indicating similar or even identical CRP ligands in both polysaccharides (Fig. 2). Since no differences in the binding reactivity between the proteinaceous and the alkali-treated HPG preparations could be observed, the latter one was used for the subsequent tests.

In the quantitative precipitin test, CRP reacted with HPG yielding typical optimum curves with the point of maximum precipitation lying in the zone of CRP excess. However, there was no reaction with larch arabinogalactan, which has a similar arrangement of the galactosyl groups.

Precipitation inhibition assay

The results of quantitative precipitation inhibition studies are shown in Fig. 3 and in Table 1. Not one of the indicated monosaccharides, with the exception of *N*-acetyl neuraminic acid, had any inhibitory effect on the precipitation of HPG by CRP. On the contrary, each of the tested substances containing monoesterified phosphate groups reduced substantially the precipitin reaction. Phosphorylcholine (PC) was found to be the most potent inhibitor, producing 50% inhibition at a concentration of 15·25 μM. The amount of each compound required for 50% inhibition and its relative inhibitory power compared with phosphorylcholine are shown in Table 1. The second-best inhibitor, *p*-nitrophenyl phosphate, was considerably less active, with a relative inhibitory power of 0·21. Phosphorylethanolamine, a structural analogue of PC that has no quarternary ammonium group, and the other phosphate monoesters listed were even less potent inhibitors of the CRP:HPG interaction. Whereas the choline base was not inhibitory up to 10 mM, inorganic phosphate was found to be a moderate inhibitor

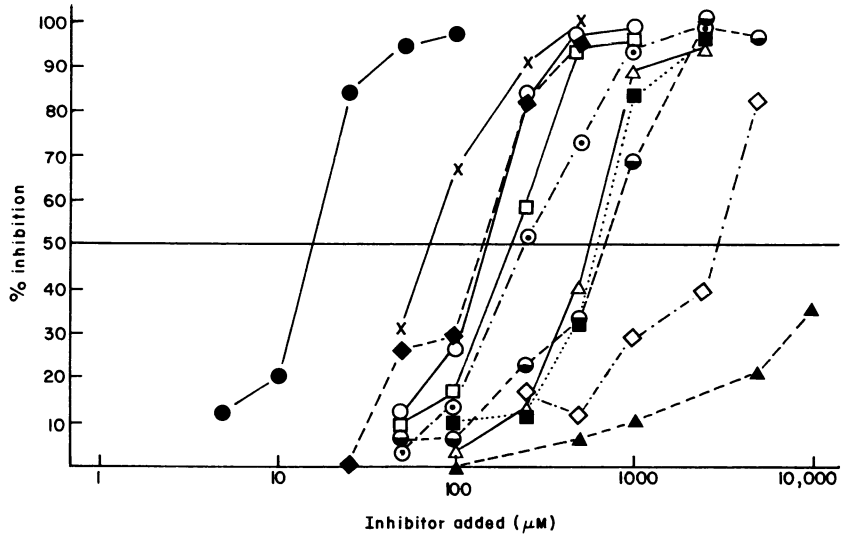

Figure 3. Inhibition by increasing amounts of inorganic phosphate and phosphate monoesters of the precipitin reaction between CRP (25 μg) and *Helix pomatia* galactan (10 μg): (●——●) phosphorylcholine, (×——×) *p*-nitrophenyl phosphate, (♦——♦) phosphorylethanolamine, (○——○) guanosine 5′ monophosphate, (□——□) D-galactose-6-phosphate, (⊙- - -⊙) D-ribose-5-phosphate, (△——△) creatine phosphate, (■· · · · · ■) β-glycerophosphate, (◒- - -◒) D-glucose-6-phosphate, (◊- · -◊) α-D-galactose-1-phosphate, (▲- - -▲) potassium dihydrogen phosphate.

Table 1. Inhibition by low molecular weight substances of the precipitin reaction between CRP and *Helix pomatia* galactan

Substance*	Concentration required for 50% inhibition [μM]	Relative inhibitory power†
phosphorylcholine	15·25	1
p-nitrophenyl phosphate	72·16	0·21
phosphorylethanolamine	141·3	0·108
guanosine 5′ monophosphate	144·0	0·106
D-galactose-6-phosphate	207·3	0·074
D-ribose-5-phosphate	241·7	0·063
creatine phosphate	573·2	0·027
β-glycerophosphate	619·0	0·025
D-glucose-6-phosphate	694·5	0·022
α-D-galactose-1-phosphate	2222·0	0·007
potassium dihydrogen phosphate	< 50% inhibition when 10 mM tested	
D-NeuNAc	14·2% inhibition when 10 mM tested	
D-Gal, D-Glc, D-Man, D-GalNAc, D-GlcNAc, L-Fuc, D-Ara, β-D-Gal-(1→3)-D-Ara‡ lactose, choline, disodium sulphate	not inhibitory up to 10 mM	

* NeuNAc, *N*-acetyl neuraminic acid; Gal, galactose; Glc, glucose; Man, mannose; GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine; Fuc, fucose; Ara, arabinose.

† Molar ratio of phosphorylcholine to inhibitor compound at 50% inhibition.

‡ Tested up to 5·4 mM.

requiring a concentration exceeding 10 mM for 50% inhibition. Free sulphate had no inhibitory effect at all.

Characterization of galactan-bound phosphate groups

Helix pomatia galactan contains about 1·8% (by weight) covalently bound phosphate, which could not be removed by treatment with 20% potassium hydroxide or by incubation with two alkaline phosphatases from different sources. In contrast, after treatment with 48% hydrofluoric acid at 4°, a procedure which selectively cleaves phosphate ester linkages, the galactan was no longer precipitated by CRP (Table 2). The same effect was observed with *Helix aspersa* galactan and with *Streptococcus pneumoniae* C polysaccharide. This inactivation of the ligand properties of HPG proceeded rather fast, since less than 4 hr of incubation with HF were sufficient to stop completely the precipitin reaction (Fig. 4).

By means of gel filtration on Biogel A-5m, a partial degradation of HPG by the action of HF could be observed. Nevertheless, this polysaccharide preparation was still precipitated by the D-galactose-specific lectins from *Ricinus communis* (RCA-1) and *Tridacna maxima*, forming somewhat diffuse precipitation lines in agarose gels. However, even after an incubation with HF for 36 hr, no liberated phosphate could be detected in the HPG samples. Therefore, the HF-treated galactan was analysed by gel filtration on Sephadex G-25 in order to find out whether phosphate had been split off in ester bound form. As Fig. 5 shows, the total carbohydrate was exclusively found in the void volume, whereas the total phosphate but no carbohydrate eluted with the salt volume of the column. Since only bound phosphate but no inorganic phosphate could be detected, evidently a phosphate-containing compound is split off by HF action. Although phosphorylcholine turned out to be the best inhibitor of the CRP:HPG interaction (Table 1), it was not possible to detect any choline in HPG or in the liberated phosphate ester (subsequently called X-P). Furthermore, the galactan was not precipitated by the PC-binding mouse mye-

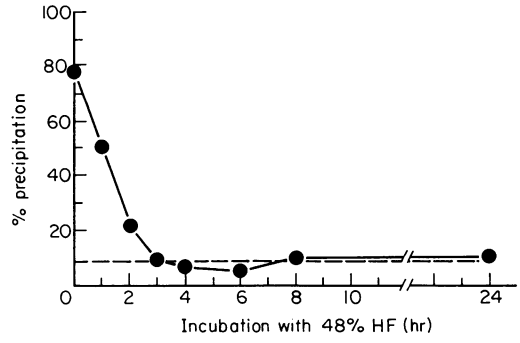

Figure 4. Influence of the treatment of *Helix pomatia* galactan with hydrofluoric acid on the precipitin reaction with CRP. The percentages of precipitate formed in comparison with the reaction with unaffected galactan are given.

Table 2. Influence of the treatment of polysaccharides with hydrofluoric acid (HF) on the precipitin reaction with CRP

Experiment*		Protein concentration of the precipitate [μg/ml]‡
I	CRP + TBS-Ca^{2+} buffer	8·5
II	CRP + *Helix pomatia* galactan	167·0
III	CRP + *Helix pomatia* galactan, HF treated	8·0
IV	CRP + *Helix aspersa* galactan	117·5
V	CRP + *Helix aspersa* galactan, HF treated	8·5
VI	CRP + *Streptococcus pneumoniae* C polysaccharide	229·0
VII	CRP + *Streptococcus pneumoniae* C polysaccharide, HF treated	3·0

* HF treated: treatment with 48% hydrofluoric acid at 4° for 24 h.

† Mean of two experiments.

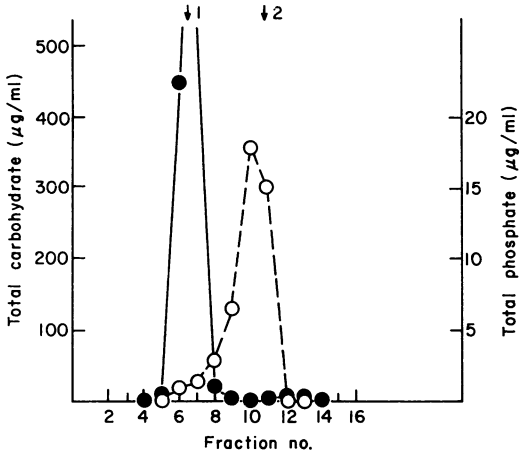

Figure 5. Gel filtration of hydrofluoric acid-treated *Helix pomatia* galactan (reaction mixture after removal of HF) on Sephadex G-25 in distilled water: (●——●) total carbohydrate, (○- - -○) total phosphate. 1 = position of blue dextran, 2 = position of dinitrophenyl L-alanine.

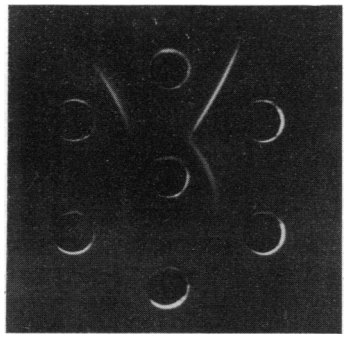

Figure 6. Double diffusion test in agarose gel. Substances from 12 o'clock (= 1) are clockwise: 1 = CRP 1% in 3% citrate, 2 = *Streptococcus pneumoniae* C polysaccharide, 6 = *Helix pomatia* galactan, centre well = mouse myeloma protein TEPC 15 in ascites. The polysaccharides were tested at 1% in 0·01 M TBS-Ca^{2+} buffer.

loma protein TEPC 15, which did react with streptococcal C polysaccharide (Fig. 6). Neither sugars nor protein were found to be constituents of X-P. Since no inorganic phosphate could be detected after incubating X-P in 0·1 M hydrochloric acid at 100° for 30 min, X-P cannot consist of pyrophosphate either.

Finally, *Helix pomatia* galactan was examined by means of gas liquid chromatography for the presence of sugar phosphate residues. However, even after mild hydrolysis of HPG to prevent phosphate ester cleavage, no monosaccharide phosphates could be detected.

DISCUSSION

The marked elevation CRP serum levels following infection or tissue injury resulting from trauma, burns or a variety of inflammatory diseases and the antibody-like *in vitro* reactivities of the protein suggest that CRP may be actively involved in non-specific host defence mechanisms (Pepys & Baltz, 1983). Since most of these reactivities with potential biological relevance (e.g. complement activation or binding to blood cells) are only exhibited after aggregation of CRP by physical means or by association with specific ligands (reviewed by Pepys & Baltz, 1983), a better understanding of the protein's binding specificities and the identification of physiological ligands seems to be crucial for revealing the biological roles of CRP.

In this connection, we have studied the so far unknown molecular basis for the *in vitro* interaction of CRP with snail galactans (Uhlenbruck *et al.*, 1979, 1981). Since most of these polysaccharides, including the structurally best-characterized *Helix pomatia* galactan, are homogalactans consisting almost exclusively of D-galactose (and to a smaller extent L-galactose) residues (Duarte & Gorin, 1983), we earlier suggested that CRP may have an additional lectin-like binding reactivity for sugar residues (Uhlenbruck *et al.*, 1979). Considering the wide distribution of galactose-containing antigens, as a lectin, CRP should recognize a considerably broader spectrum of harmful material in the blood stream. In the present study, however, we could not obtain evidence to suggest that CRP binds carbohydrate residues. There was no alteration of the UV absorbance of CRP by *o*-nitrophenyl β-D-galactopyranoside, nor could galactose or any other simple sugar tested inhibit the precipitin reaction between CRP and HPG. The low inhibitory effect of N-acetyl neuraminic acid is probably only due to the acid nature of this sugar.

On the other hand, we observed a strong inhibition by a high concentration of inorganic phosphate in agar gel diffusion test and by a number of phosphate monoesters when using the microprecipitin test. It seems paradoxical that phosphorylcholine was the most potent inhibitor, although this compound could not be detected in HPG. The results show that CRP binds in the presence of calcium ions to phosphate residues on HPG, whereas there is no precipitin reaction with larch arabinogalactan which does not contain phosphate. The complete loss of CRP receptor properties of HPG by the treatment with hydrofluoric acid under conditions that are used for the cleavage of phosphate esters (Mort & Lamport, 1977) also strongly indicates that CRP may bind to phosphate groups that are minor consituents of HPG and other snail galactans. In contrast to Weinland (1956), who identified by means of paper chromatography phosphate-containing sugar compounds in HPG hydrolysates, we have not detected any sugar-bound phosphate. This, and the fact that no inorganic phosphate but a compound containing covalently bound phosphate is split off by treatment of HPG with hydrofluoric acid, strongly indicates that phosphate groups are attached to a as yet unidentified non-carbohydrate substance, which links the phosphate to the carbohydrate chains in the intact galactan molecule. Therefore, it seems possible that CRP does not merely bind to the phosphate groups on the HPG molecule, but that the actual ligand comprises the whole linkage group released by hydrofluoric acid. Support for this assumption comes from the observation that phosphate monoesters were more potent inhibitors of the CRP:HPG interaction than inorganic phosphate.

In this connection, it is worth mentioning that the binding reaction between the CRP-related human serum amyloid P protein (SAP) and agarose is not based on an interaction of this protein with D-galactose residues as suggested previously (Uhlenbruck *et al.*, 1979). Rather, SAP binds to the cyclic 4,6-pyruvate acetal of D-galactose but the binding is not inhibited by

D-galactose, pyruvate or methyl β-D-galactopyranoside (Hind *et al.*, 1984).

In summary, our results show that the anti-galactan reactivity of human *C*-reactive protein is not based on galactose-binding properties, as it was demonstrated for several anti-galactan lectins (Eichmann, Uhlenbruck & Baldo, 1976), but that this binding reactivity is obviously a variant form of the anti-phosphate/anti-phophorylcholine specificity of CRP. On the other hand, the binding of C-reactive protein to pneumococcal type 4 polysaccharide (Heidelberger *et al.*, 1972) and agarose (Pepys *et al.*, 1977) cannot be explained by this antiphosphate reactivity.

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