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Isolation and characterization of C-reactive protein and serum amyloid P component in the rat

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Summary. C-reactive protein (CRP) and serum amyloid P component (SAP) have been identified for the first time in rat serum and isolated by calcium-dependent affinity chromatography. Rat CRP closely resembled human CRP in its amino acid composition, in having five subunits per molecule and in its electron microscopic appearance as a pentameric annular disc. It differed, however, from all other mammalian CRP's characterised hitherto in being a glycoprotein bearing a single complex oligosaccharide on each polypeptide subunit. Furthermore one pair of its subunits per molecule was linked by interchain disulphide bridges whereas in other animals the subunits of both CRP and SAP are all non-covalently associated.

The serum concentration of CRP in normal healthy laboratory rats and in specific pathogen-free rats was 300–600 μg/ml which is much greater than has been described in any other species and exceeds even maximal acute phase levels of CRP in man. Following injections of casein or croton oil, serum CRP levels rose to a maximum of about 900 μg/ml. Rat CRP bound to pneumoccocal C-polysaccharide (CPS) but, in marked contrast to the behaviour of CRP from man, rabbit and marine teleost fish, it did not precipitate with CPS solutions, agglutinate CPS-coated sheep erythrocytes or initiate complement activation.

Rat SAP, like SAP of other species, was a glycoprotein but unlike them it was composed only of a single pentameric disc not two such discs interacting face-to-face. The normal level of SAP in rat serum was 20–50 μg/ml, very similar to the levels seen in man, and it did not behave as an acute phase reactant in response to casein or croton-oil injections. In this respect it resembled human SAP but differed from murine SAP which is a major acute phase reactant.

Abbreviations: AP, amyloid P component; CFT, complement fixation test diluent; CPS, pneumoccocal C-polysaccharide; CRP, C-reactive protein; E-CPS, sheep erythrocytes coupled with pneumoccocal C-polysaccharide; PAGE, polyacrylamide gel electrophoresis; SAP, serum amyloid P component; SDS, sodium dodecyl sulphate; PAS, periodic acid-Schiff stain, CoF, cobra factor.

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INTRODUCTION

C-reactive protein (CRP) and serum amyloid P component (SAP) are closely related plasma proteins in man. The whole amino acid sequence of CRP is known and, despite minor similarities, it has no statistically significant homology with any protein other than SAP (Oliveira, Gotschlich & Liu, 1977, 1979). Of the

known sequence of SAP about 60% is homologous with CRP (Osmand, Friedenson, Gewurz, Painter, Hofmann & Shelton, 1977; Painter, Pinteric, Hofmann, Kells & Katz, 1976; Pepys, Dash, Munn, Feinstein, Skinner, Cohen, Gewurz, Osmand & Painter, 1977b; Levo, Frangione & Franklin, 1977; Skinner, Pepys, Cohen, Keller & Lian, 1980) and the two proteins also have a similar appearance in the electron microscope, consisting of subunits arranged in a disc-like configuration with cyclic pentameric symmetry (Osmand *et al.*, 1977; Painter *et al.*, 1976; Pepys *et al.*, 1977b). Both proteins have the capacity for calcium-dependent ligand binding, albeit to different ligands (Pepys *et al.*, 1977b; Abernathy & Avery, 1941; Pepys, Dash & Ashley, 1977a). Using this property, we have isolated from the serum of a range of vertebrate species, proteins with the same ligand specificity as human SAP and CRP and with similar electron microscopic appearances and subunit composition (Pepys, Dash, Fletcher, Richardson, Munn & Feinstein, 1978a). We have established by amino-terminal amino acid sequencing that CRP and SAP from the plaice (*Pleuronectes platessa* L.), a marine teleost, are indeed homologues of the human proteins (Pepys, Baltz, Dyck, de Beer, Evans, Feinstein, Milstein, Munn, Richardson, March, Fletcher, Davies, Gomer, Cohen, Skinner & Klaus, 1980).

In addition to the inherent interest in phylogeny of this protein family, which has been stably conserved throughout vertebrate evolution, CRP is significant because it is the classical acute phase reactant in man (Tillett & Francis, 1930; Abernathy *et al.*, 1941; Pepys, 1981) whilst SAP is apparently identical with amyloid P component (AP; Pepys *et al.*, 1977b; Skinner *et al.*, 1980), a universal constituent of all forms of amyloid deposits (Pepys, 1981). We report here for the first time the isolation and characterization of CRP and SAP in the rat. Rats have been widely used for studies of the acute phase response (Koj, 1974; Gordon, 1976) and are exceptionally resistant to the deliberate induction or spontaneous development of amyloidosis (Dunn, 1967; Jakob, 1971; Green, 1974).

MATERIALS AND METHODS

Rat serum

Serum was obtained from conventional Wistar rats bred at the RPMS, and specific pathogen-free Wistar rats supplied by the Joint Animal Breeding Unit, University of Nottingham, Loughborough, Leics. Sera from locally bred August and Lister Hooded rats were provided by Mr Derek Simmonds, Institute of Cancer Research, Pollards Wood.

Antisera

Antisera to isolated rat SAP and CRP were raised by immunisation of Sandy Lop rabbits (Hylyne Ltd, Cheshire) with the purified proteins emulsified in Freund's complete adjuvant (Difco Laboratories, Surrey), followed by booster injections emulsified in Freund's incomplete adjuvant (Difco). Specificity of antisera was tested by double diffusion in 1% agarose (Indubiose A37, IBF, Clichy, France) gel in 0·075 M veronal buffer/0·01 M ethylenediamine tetracetic acid (EDTA) pH 8·6, and by electroimmunoassay (see below). Monospecific rabbit anti-rat C3 serum was raised by a modification of the method of Mardiney & Müller-Eberhard (1965).

Affinity chromatography

Calcium-dependent affinity chromatography of SAP (Pepys *et al.*, 1977a) was performed using either Sepharose 6B gel (Pharmacia (G.B.) Ltd, Hounslow, Middlesex) or Miles LE agarose (Miles Labs Ltd, Stoke Poges). A gel of 4% w/v Miles agarose was prepared in 0·01 M Tris/0·14 M NaCl/0·002 M $CaCl_2$ pH 8·0 (Tris/Ca/saline) and was homogenized in a laboratory blender at 12,000 r.p.m. (MSE Ato-Mix, C. E. Payne & Sons, London). Pneumococcal C-polysaccharide (CPS) was prepared and coupled to cyanogen bromide-activated Sepharose 4B as previously described (Pepys *et al.*, 1977b). For isolation of specific ligand-binding proteins, serum was passed over the appropriate gel which had been equilibrated in Tris/Ca/saline and the gel was washed in this buffer until no material absorbing at 280 nm was eluted. The absorbed protein was then eluted using 0·01 M Tris/0·01 M EDTA/0·14 M NaCl pH 8·0 (Tris/EDTA/saline), and was subjected to further purification steps as required (see below and results). Human CRP and SAP were isolated as previously described (Pepys *et al.*, 1977a, b). The IgG fraction of specific rabbit anti-rat CRP serum was isolated on a column of Sepharose-protein A (Pharmacia) according to the manufacturer's instructions. Twenty-seven milligrams of this IgG was coupled to 7 ml of cyanogen bromide-activated Sepharose 4B gel (Pharmacia) to provide an insoluble absorbent which was used to remove traces of rat CRP from the rat SAP preparations.

Gel filtration
A 2·6 × 80 cm column of Sephacryl S-300 (Pharmacia) was calibrated with standard globular proteins of known molecular weight (Pharmacia) as follows: bovine thyroglobulin, 669K; horse spleen ferritin, 440K; bovine liver catalase, 232K; rabbit muscle aldolase, 158K; bovine serum albumin, 67K; hen egg ovalbumin, 43K; bovine pancreatic chymotrypsinogen A, 25K and bovine pancreatic ribonuclease A, 13·7K. Five millilitre samples were applied and eluted with Tris/EDTA/saline at 16 ml/hr. Normal rat serum diluted 1:3 in Tris/EDTA/saline was run on this column and the eluted fractions assayed for CRP and SAP as described below. Isolated human CRP and SAP were also run on the same column.

Polyacrylamide gel electrophoresis (PAGE)
The apparent molecular weights of isolated rat CRP and SAP were estimated by electrophoresis in 4%–30% gradient polyacrylamide gels (PAA 4/30, Pharmacia) run in non-dissociating conditions (Tris/borate/EDTA buffer pH 8·4). Standard globular proteins of known molecular weight (Pharmacia) were used as markers for the construction of a standard curve: hog thyroglobulin, 669K; horse spleen ferritin, 440K; beef liver catalase, 232K; beef liver lactate dehydrogenase, 140K and bovine serum albumin, 67K. Isolated human SAP and CRP were also run in the same gels. The apparent molecular weights of the subunits of rat CRP and SAP were estimated by electrophoresis in 12% polyacrylamide gels containing sodium dodecyl sulphate (SDS, Sigma Chemical Co. Ltd, Surrey) run under both reducing and non-reducing conditions (Laemmli, 1970). Samples run under non-reducing conditions contained 50 mM iodoacetamide (final concentration) to prevent disulphide interchange or oxidative disulphide cross-linking of separate subunits. Standard proteins of known molecular weight were run as markers for the construction of a standard curve: rabbit muscle phosphorylase b, 94K; bovine serum albumin, 67K; hen egg ovalbumin, 43K; bovine erythrocyte carbonic anhydrase, 30K; soy bean trypsin inhibitor, 20·1K; bovine milk α-lactalbumin, 14·4K. Isolated human SAP and CRP were also run in the same gels.

Isoelectric focusing
Isolated rat SAP and CRP were run in LKB Ampholine PAG plates pH 3·5–9·5 on the LKB Multiphor apparatus according to the manufacturer's instructions (LKB Instruments Ltd, London).

Ultracentrifuge studies
Isolated rat CRP at 0·83 mg/ml in 0·1 M Tris/0·01 M EDTA buffer pH 8·0 was subjected to sedimentation equilibrium analysis in a Beckman model E ultracentrifuge using the high speed meniscus depletion method of Yphantis (1964). Rotor speeds of 13,000 and 17,000 r.p.m. were used and sedimentation followed by Raleigh interference optics. A partial specific volume of 0·73 cm^3/g was assumed in calculating the molecular weight.

Characterization of rat SAP and CRP as glycoproteins
SDS-PAGE slabs in which isolated rat CRP and rat SAP, human CRP and human SAP had been run in duplicate were divided into two replicate halves. One was stained for protein as usual with Coomassie Blue and the other was stained for carbohydrate by the periodic acid-Schiff (PAS) stain as described elsewhere (Glossmann & Neville, 1971). Isolated rat CRP and SAP were also tested in the anthrone reaction for total hexoses (Roe, 1955). Bovine serum albumin and human CRP, neither of which is glycosylated, and rabbit IgG, a known glycoprotein, were tested in parallel.

Amino acid and carbohydrate analysis of rat CRP
Portions of the same sample of rat CRP were subjected respectively to carbohydrate analysis as described elsewhere (Clamp, 1977) and to amino acid analysis as follows. Duplicate aliquots were hydrolysed *in vacuo* at 110° or 130° for between 16 hr and 108 hr with 6 M HCl (Aristar Grade) to which a crystal of phenol had been added to protect tyrosine side chains against oxidation and containing norleucine as internal standard. Hydrolysates were analysed on a Beckman amino acid analyser model 121 MB (Spackman, Stein & Moore, 1958). Figures for serine and threonine were extrapolated to zero time and for leucine, isoleucine and valine to infinite time ($1/t = 0$). All figures were corrected for the observed recovery of norleucine. Cysteine was determined by the incorporation of radioactivity from 1-[^{14}C] iodoacetic acid. Tryptophan was determined spectrophotometrically (Beaven & Holiday, 1952). The N-terminal residue was sought by dansylation of isolated rat CRP under denaturing conditions followed by acid hydrolysis (Gray, 1967).

Electron microscopy
Two separate preparations of each protein were examined using the negative staining technique. Both the following procedures were used, with cold solu-

tions in a +4° laboratory. Droplets of solutions of the proteins at various concentrations were placed on holey-carbon films (Huxley & Zubay, 1960) on 200 mesh grids. The specimens were allowed to absorb for 30 sec to 1 min then excess liquid was withdrawn with filter paper and replaced with a drop of 3% (w/v) sodium silicotungstate, pH 7·2. This in turn was withdrawn and the grids then allowed to dry. Preparations were also negatively stained with 3% sodium silicotungstate by the method of Valentine, Shapira & Stadtman (1968) modified by picking up the thin carbon film from the negative staining solution on a grid coated with a holey-carbon film. Most detail was visible in molecules lying over the holes.

Precipitation by CRP

Fifty-microlitre volumes of various concentrations of CPS and of rat or human CRP (maximum concentration 2·5 mg/ml) in Tris/Ca/saline were mixed to provide weight for weight ratios ranging from sixty-four-fold excess of CPS to sixty-four-fold excess of CRP. Tubes were incubated at 37° for 30 min then at 4° overnight and precipitation estimated by resuspending the contents and assessing the degree of tubidity by eye. Precipitation by unseparated CRP in rat serum was sought in comparable tests and by double diffusion against various concentrations of CPS in agarose gel in Tris/Ca/saline.

Agglutination by CRP

CPS was coupled to sheep erythrocytes by the benzoquinone method (Ternynck & Avrameas, 1976). Two milligrams of lyophilized CPS dissolved in 400 μl of phosphate-buffered saline pH 7·4 were mixed with 3 mg of benzoquinone (Quinone AR 69258, Koch-Light Ltd, Colnbrook, Bucks.) dissolved in 100 μl of ethanol. The mixture was rotated in the dark for 1 hr at 21° before separation of the activated CPS by gel filtration in a column of Sephadex G25 (PD10, Pharmacia). The column was eluted with 0·1 M $NaHCO_3$, the activated CPS collected at the void volume and added to 0·4 ml of washed packed sheep erythrocytes together with 2 ml of 0·1 M $NaHCO_3$. The mixture was rotated at 37° for 30 min and the cells were then washed three times in 0·9% w/v NaCl. The CPS-coupled sheep erythrocytes (E-CPS) were preserved by fixation with 0·25% v/v glutaraldehyde (EM grade, TAAB Labs, Reading) and were suspended at 1% in Tris/Ca/saline containing 1% w/v bovine serum albumin (BSA; Sigma Chemical Co. Ltd, Surrey). Fresh normal rat serum, isolated rat CRP at 4 mg/ml and 1 mg/ml and human CRP at 1 mg/ml were titrated in doubling dilutions in 25 μl volumes in U-bottomed Microtiter plates (Sterilin Ltd, Teddington, Middx) in Tris/Ca/saline containing 1% w/v BSA. Twenty-five microlitre volumes of 1% E-CPS were added to each dilution, mixed and left to settle at 21°. Agglutination patterns were read by eye and 5 μl of 0·2 M EDTA pH 7·2 were then added to each well to give a final concentration of 0·018 M EDTA. After resuspension the plates were left to settle and the agglutination patterns were read again.

Complement activation by CRP

Fresh normal serum from a single rat was separated within 1 hr of clotting at 21° and stored in aliquots under liquid nitrogen. It contained 550 μg/ml of CRP. Three different batches of CPS were dissolved at 1 and 5 mg/ml in veronal-buffered saline pH 7·2 containing calcium and magnesium ions (complement fixation test diluent, CFT, Oxoid Ltd, London). Twenty-microlitre volumes of these CPS solutions were incubated with 20 μl volumes of the rat serum for 60 min at 37°. Positive and negative controls for complement activation included: addition of 10 μg of purified cobra factor (CoF; Pepys, Tomkins & Smith, 1979c), the C3-activating protein of cobra venom, instead of CPS; addition of 5 μl of isolated human CRP at 5 mg/ml in Tris/Ca/saline together with CPS; and addition of CFT alone instead of CPS. Activation of complement in rat serum was quantified by estimation of the electrophoretic conversion of native C3 into products of faster mobility using the crossed immunoelectrophoresis technique (Ganrot, 1972). Rabbit anti-rat C3 serum was incorporated in 1% w/v agarose (Indubiose A37) gel for the second dimension. The gel and trough buffer was 0·075 M veronal/0·01 M EDTA pH 8·6 throughout. In further experiments a batch of CPS, which together with human CRP produced marked C3 activation in rat serum, was titrated in doubling dilutions in CFT from 1 mg/ml to 1 μg/ml and 20 μl volumes of each dilution were incubated with rat serum as above. The capacity of complexes of isolated rat CRP to activate rat complement was tested by mixing 5 μl volumes of isolated rat CRP at 5 mg/ml, 2·5 mg/ml, 1 mg/ml and 200 μg/ml with 15 μl volumes of CPS at respectively 1 mg/ml, 200 μg/ml and 40 μg/ml, and then adding in 20 μl volumes of rat serum. After 60 min of incubation at 37° conversion of rat C3 was measured as above. Precisely parallel experiments using human instead of rat CRP were performed simultaneously. Controls were included in which CFT

replaced either CRP or CPS at each of the concentrations tested.

Quantification of rat CRP and SAP

Serum concentrations of CRP and SAP were estimated by electroimmunoassay (Laurell, 1972) using rabbit anti-rat CRP and SAP sera incorporated in 1% w/v agarose (Indubiose A37, IBF, Clichy, France) gel in 0·075 M veronal buffer pH 8·6 containing 0·01 M EDTA. The troughs contained the same buffer and electrophoresis was performed for 6 hr at 6 V/cm. The assays were calibrated using samples of the isolated pure proteins.

Induction of acute phase responses

Groups of five adult male and female rats were individually numbered and bled from the tail before receiving subcutaneous injections of either 0·5 ml of 1% v/v croton oil (Sigma) in liquid paraffin or 1·5 ml of 10% w/v casein (Janigan, 1965)—BDH Chemicals Ltd, Poole, Dorset. Each animal was then bled daily for the next 6 days, the sera separated individually and stored at −20° before being assayed in one batch at the end of the experiment.

RESULTS

Isolation of rat CRP

A typical preparation of rat CRP (Table 1) was started with 100 ml of pooled serum from normal adult Wistar rats. This was passed over a column of Sepharose-CPS, bearing approximately 50 mg of CPS, equilibrated with Tris/Ca/saline. The column was washed with the same buffer until no further material absorbing at 280 nm was detected and was then eluted with Tris/EDTA/saline. All eluted protein was pooled, and dialysed back into Tris/Ca/saline. This crude CRP contained some SAP, which was removed by passing it over a 60 ml column of Sepharose 6B in Tris/Ca/saline. The non-absorbed CRP was pooled, concentrated and finally isolated by gel filtration in Tris/EDTA/saline on Sephacryl S-300 (Fig. 1).

Isolation of rat SAP

Two 100-ml lots of rat serum were sequentially absorbed onto a 60 ml batch of Sepharose 6B equilibrated with Tris/Ca/saline. After washing to remove all non-absorbed protein, SAP was eluted from the Sepharose with Tris/EDTA/saline and the two separate eluates were pooled. Contaminating CRP was absorbed in the presence of EDTA using rabbit anti-rat CRP antibodies insolubilized on cyanogen bromide-activated Sepharose 4B. A yield of 1·3 mg of protein was obtained, and the SAP content of the starting material was subsequently found to have been 5 mg.

Unfortunately all recent lots of Sepharose beads from Pharmacia have a lower capacity for binding SAP than those used in our earlier work (Pepys *et al.*, 1977a; Pepys, 1979), and an alternative procedure for isolation of rat SAP was therefore devised. CRP was first absorbed out of rat serum using the Sepharose-CPS column. Some SAP was also unavoidably removed by calcium-dependent binding to the Sepharose matrix, but the remainder, largely free of CRP, was then isolated using Miles LE agarose gel, followed by a final absorption in the presence of EDTA with insolubilized anti-rat CRP. Rat CRP has a much greater tendency to undergo calcium-dependent binding to agarose than does human CRP (Pepys *et al.*, 1977b). If prior removal of rat CRP on the CPS column was omitted, large amounts of CRP bound to and eluted from the Miles agarose, thereby heavily contaminating the SAP.

Table 1. Isolation of rat CRP

	Protein content* (mg)	CRP content† (mg)	Purity of CRP (%)
Starting serum (100 ml)	ND	60·0	ND
Eluate from Sepharose-CPS	49·7	44·1	88·7
After absorption with Sepharose	35·0	33·9	96·9
After gel filtration on Sephacryl S-300	28·0	28·0	100·0

* Measured by A_{280} assuming $E^{1\%}_{1cm}$ of rat CRP = 17·0, which is the value measured by interferometry for human CRP and SAP and mouse SAP (unpublished observations).

† Measured by electroimmuno assay.

ND, not done.

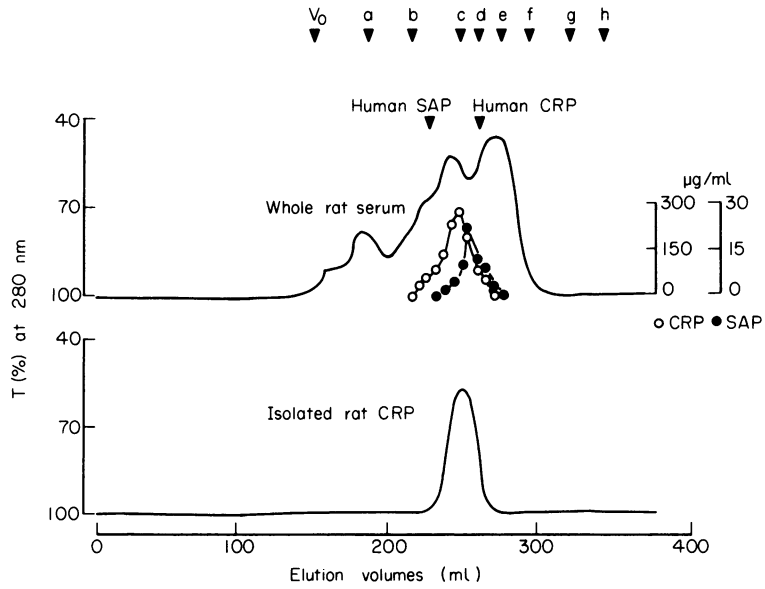

Figure 1. Gel filtration of isolated rat CRP (below) and whole rat serum (above) on a calibrated column of Sephacryl S-300. The solid lines show the transmittance (%) of the column effluent at 280 nm and the elution volumes for globular marker proteins of known molecular weight and for human SAP and human CRP are arrowed; a, 669K; b, 440K; c, 232K; d, 158K; e, 67K; f, 43K; g, 25K; h, 13·7K. The peak heights in electroimmunoassay for CRP (o) and SAP (•) in the eluted fractions of rat serum are also shown.

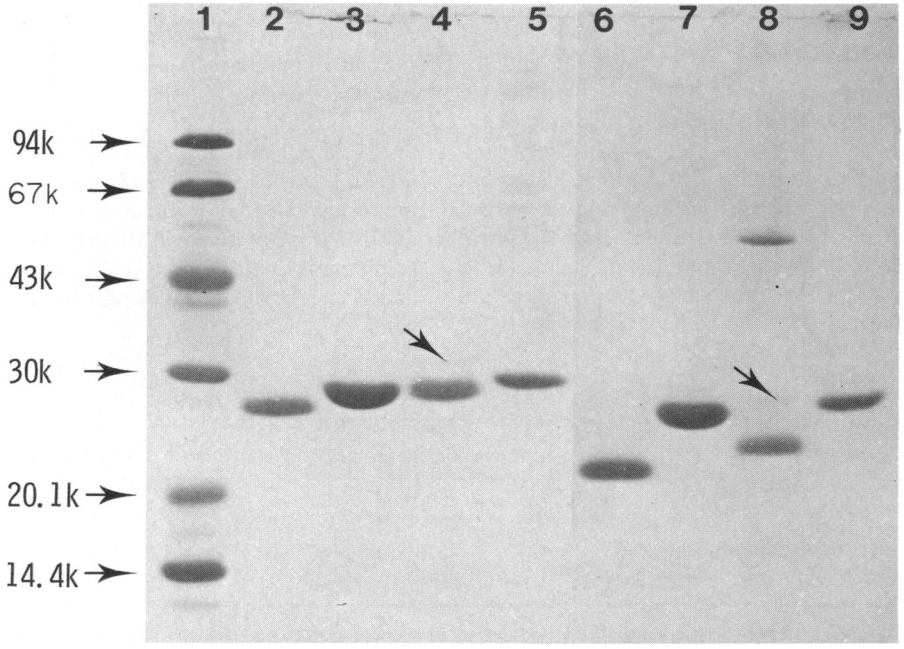

Figure 2. SDS-12% PAGE of rat CRP and SAP. Track 1, marker proteins of known molecular weight; 2, reduced human CRP; 3, reduced human SAP; 4, reduced rat CRP, the position of the minor band is arrowed; 5, reduced rat SAP; 6, non-reduced human CRP; 7, non-reduced human SAP; 8, non-reduced rat CRP, the positions of the trace band is arrowed; 9, non-reduced rat SAP.

Structural characterization of rat CRP

The apparent molecular weight of rat CRP, either in serum or after isolation, determined by gel filtration on a Sephacryl S-300 column calibrated with standard globular proteins was 173,000 Daltons (Fig. 1). Human CRP and human SAP run on the same column had apparent mol. wt of 138,000 and 316,000, respectively. However when the calibration curve of K_{av} *v.* log molecular weight was normalised to the true mol. wt of human CRP, 105,500 Daltons, derived from sequence data (Oliveira *et al.*, 1977; 1979), the apparent mol wt of human SAP and of rat CRP became 234,000 and 129,000 Daltons, respectively. The value for human SAP corresponds closely with estimates previously derived by sedimentation equilibrium analysis (Painter *et al.*, 1976; Pepys *et al.*, 1978a) and the same was true for rat CRP (see below). The anomalously large apparent molecular weights of these proteins when compared with globular protein markers are probably a result of their known molecular asymmetry.

After reduction with mercaptoethanol 95% of the protein from purified preparations of rat CRP migrated in SDS-12% PAGE with an apparent mol. wt of 29,000 and 5% of it ran at 33,300 using a calibration curve derived from standard marker proteins (Fig. 2). The subunits of human CRP are known to run with an anomalously large apparent molecular weight in this SDS-PAGE system (Pepys *et al.*, 1978a), for reasons which are not presently understood, and the calibration curve was therefore normalized according to the position of human CRP (subunit 21,100) run in the same gel (Fig. 2). Using this normalized curve the apparent mol. wt of the major band from rat CRP was 23,100 and that of the trace band was 26,500. In the same gel rat CRP ran under non-reducing conditions and in the presence of iodoacetamide gave three bands of which one comprising 55% of the protein had an apparent mol. wt of 23,200 (using the normalized calibration curve, 18,500), another comprising 5% of the protein ran at 28,300 (22,500) and the third comprising 40% of the protein was at 57,000 (45,100)—Fig. 2.

In the electron microscope rat CRP was apparently composed of five subunits arranged in an annular disc-like configuration with cyclic pentameric symmetry (Fig. 3a). The maximum diameter of the molecules viewed face-on was 10 nm and the width of the subunits in the radial plane of the whole molecule was 3·3 nm. These appearances and dimensions were the same as for CRP molecules from man and other

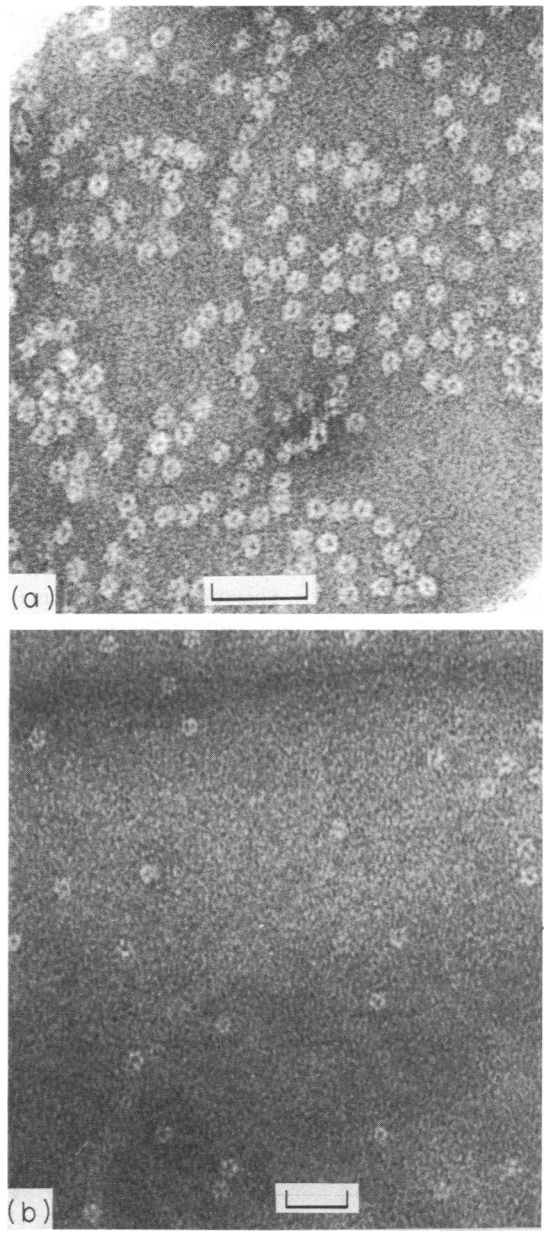

Figure 3. Electron micrographs of rat CRP(a) and SAP(b) negatively stained with 3% sodium silicotungstate. The scale bars represent 30 nm.

mammals. Structures interpreted as being edge-on views of the rat CRP molecules showed that the discs occurred both singly and in pairs in about equal proportions. A very small number of stacks, containing up to seven discs, were seen.

The molecular weight of isolated rat CRP estimated directly by sedimentation equilibrium analysis in the analytical ultracentrifuge using the high speed meniscus depletion method was 137,700 at 13,000 r.p.m. and 138,980 at 17,000 r.p.m. (Fig. 4). These results confirm that the rat CRP molecule is composed of five subunits with mol. wt of about 28,000 and the amino acid and carbohydrate composition was calculated on that basis.

Three separate preparations of isolated rat CRP run on a 4%–30% gradient PAGE under non-dissociating conditions gave a consistent pattern of four distinct bands (Fig. 5) which, when compared with the migration of standard globular protein markers, had apparent mol. wt of 138,000 (55% of the protein), 149,000 (45%), 130,000 and 160,000 (5% each). When fractions from the ascending limb, apex and descending limb of the peak of rat CRP eluted from Sephacryl S-300 were run on gradient gels they yielded identical patterns in the distribution of staining intensity between these four bands. This suggests that they may be derived artefactually during the running of the gel rather than reflecting genuinely distinct species of different molecular weight amongst the population of rat CRP molecules. In such gradient gels, just as in gel filtration, the asymmetric human CRP molecule runs with an anomalously large molecular weight compared with standard globular protein markers (Pepys *et al.*, 1978a). When the calibration curve derived from the latter was normalized to the known molecular

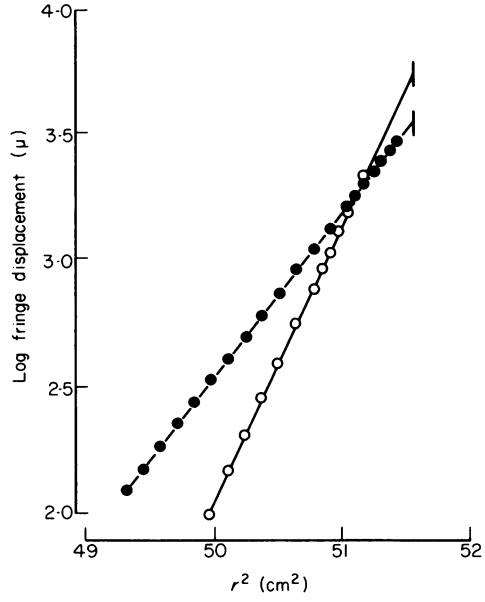

Figure 4. Sedimentation equilibrium data for rat CRP. (•) 13,000 r.p.m.; (o) 17,000 r.p.m.

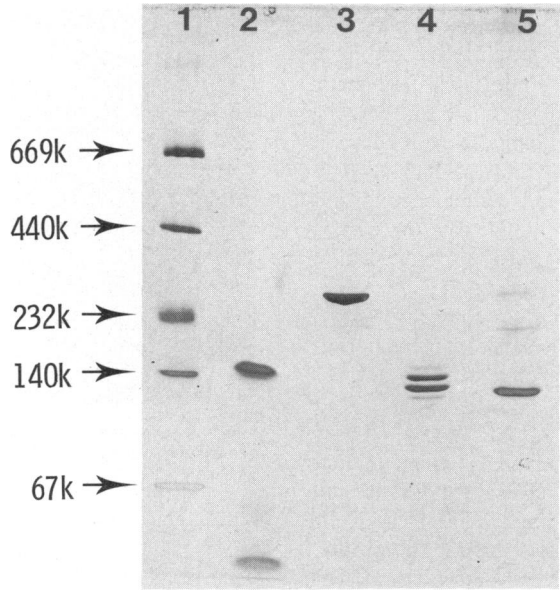

Figure 5. Four 30%-gradient PAGE of rat CRP and SAP. Track 1, marker proteins of known molecular weight; 2, human CRP; 3, human SAP; 4, rat CRP; 5, rat SAP.

weight of human CRP run in the same gel the main band of rat CRP had an apparent weight of 94,400 Daltons.

On isoelectric focusing in polyacrylamide gel, CRP isolated from pooled Wistar (outbred) rat serum produced a pattern of closely spaced bands in the pH range 3·8–4·0 (Fig. 6).

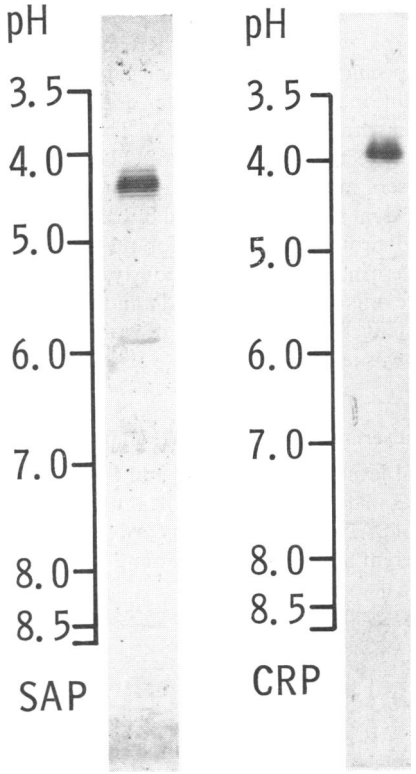

Figure 6. Isoelectric focusing in polyacrylamide gel of rat CRP and SAP. Samples were applied 1 cm from the cathode wick.

Amino acid composition of rat CRP

The amino acid composition of rat CRP is shown in Table 2 and is evidently very similar indeed to that of human CRP (Oliveira *et al.*, 1977; 1979). No free N-terminal residue was detectable indicating that it must be blocked. Preliminary mass spectrometric analysis of permethylated rat CRP suggests that the N-terminus may be acetyl alanine (Dell A., Taylor J., Bruton C., de Beer F.C. and Pepys M.B. unpublished).

Table 2. Amino acid composition of rat and human CRP

	Residues/mol	
	Rat CRP	Human CRP*
1/2 Cys	2	2
Asp	17	15
Thr	8	12
Ser	19	18
Glut	22	20
Pro	8	11
Gly	18	14
Ala	14	9
Val	19	17
Met	2	2
Ileu	12	9
Leu	16	14
Tyr	6	7
Phe	13	12
Lys	10	12
His	4	2
Arg	6	6
Try	4	5
Total residues	200	187

* Based on sequence data of Oliveira *et al.* (1977).

Structural characterization of rat SAP

The apparent molecular weight of SAP in rat serum measured by gel filtration on a Sephacryl S-300 column calibrated with standard globular marker proteins was 151,000 Daltons (Fig. 1) or 110,000 when normalized to the known molecular weight of human CRP run on the same column. In gradient PAGE, isolated rat SAP gave a single main band, comprising over 95% of the applied protein. The apparent mol. wt was 134,000, using globular protein markers, or 91,000 when normalized to the known molecular weight of human CRP run in the same gel (Fig. 5). In SDS-PAGE rat SAP yielded a single subunit the apparent mol. wt of which, normalized to human CRP run in the same gel, was 24,500 under reducing conditions and 21,000 under non-reducing conditions (Fig. 2). Isoelectric focusing yielded seven distinct bands in the pH range 4·1–4·5 (Fig. 6). In the electron microscope the rat SAP molecules appeared as symmetrical pentameric annular discs of comparatively low contrast (Fig. 3b). Their dimensions in face-on views were the same as those of rat CRP (see above) and of human SAP examined at the same time under

the same conditions. The appearance of rat SAP molecules viewed edge-on revealed that many of the discs were single, but some face-to-face pairs also occurred. There was no evidence of the formation of longer stacks of discs.

Glycoprotein nature of rat CRP and rat SAP

Both the major and the trace band yielded by highly purified rat CRP in SDS-PAGE stained positively with PAS, as did the subunit of rat SAP. In the same gel human SAP, a known glycoprotein, stained with PAS whilst human CRP did not. Rat CRP and rat SAP both gave positive results in the anthrone reaction as did rabbit IgG. The non-glycosylated proteins, human CRP (Gotschlich & Edelman, 1965) and bovine serum albumin were both negative in the same test. Rat CRP was found to contain three mannose residues, two galactose, three N-acetylglucosamine and two sialic acid residues per subunit, assuming a mol. wt of 28,000 per subunit. This suggests that each subunit is glycosylated with a single complex oligosaccharide.

Production of antisera and establishment of assays for rat CRP and SAP

Monospecific sheep anti-human CRP and anti-human SAP sera, and rabbit anti-mouse SAP serum gave no precipitation reactions in gel with rat serum. Rabbits

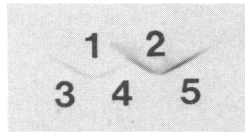

Figure 7. Immunodiffusion analysis of rabbit antisera to rat SAP and CRP. 1, rabbit anti-rat SAP serum; 2, rabbit anti-rat CRP serum; 3, isolated rat SAP (50 μg/ml); 4, normal rat serum diluted 1:2 (SAP, 19 μg/ml; CRP 275 μg/ml); 5, isolated rat CRP (500 μg/ml).

immunized with isolated rat CRP and SAP produced monospecific antisera which each gave a single precipitation line in gel against rat serum and showed no cross reaction with each other (Fig. 7). These antisera were used for electroimmunoassay of CRP and SAP levels in rat sera and to monitor subsequent isolations. A standard rat serum, established by calibration with the purified proteins, was used for reference. SAP and CRP were measured simultaneously in gels containing suitable mixtures of anti-SAP and anti-CRP antibodies (Fig. 8). The coefficient of variation of replicate assays was less than 10%.

Normal serum levels of rat SAP and CRP

Normal levels of serum CRP and SAP are shown in Table 3. There was no important difference in the levels of SAP or CRP between conventional adult rats of different strains or sexes. Young animals (3 weeks

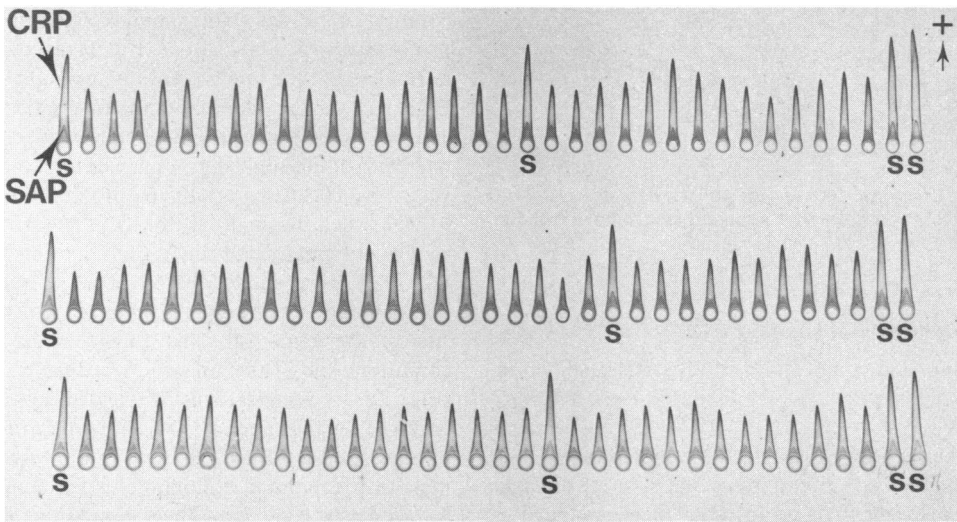

Figure 8. Electroimmunoassay of rat serum CRP and SAP in agarose gel containing a mixture of antisera to the two proteins. S, standard reference serum; the outer rocket formed by CRP and the inner rocket formed by SAP are indicated with arrows.

Table 3. Serum levels of CRP and SAP in rats

Strain	Sex	Age	Number of rats	CRP (μg/ml) Mean ± SD	CRP (μg/ml) Range	SAP (μg/ml) Mean ± SD	SAP (μg/ml) Range
Wistar	Mixed	Adult	6 pools*	422 ± 67	333–490	26 ± 6	21–34
SPF† Wistar	Female	4 Months	10	452 ± 58	365–562	31 ± 4	24–40
SPF† Wistar	Male	4 Months	10	523 ± 58	447–626	28 ± 3	26–33
August	Female	8 Months	9	411 ± 48	346–489	32 ± 4	24–37
August	Female	3 Weeks	10	96 ± 18	73–119	28 ± 5	20–38
August	Male	3 Weeks	10	196 ± 66	127–205	40 ± 7	29–50
Lister Hooded	Female	8 Months	6	536 ± 54	459–624	40 ± 6	32–49
Lister Hooded	Male	8 Months	4	601 ± 37	549–612	28 ± 2	27–32

* Six separate pools of serum from five to ten rats each.
† SPF, specific pathogen free.

old) had much lower levels of CRP than adults. Specific pathogen-free adult animals had CRP levels comparable with those of their conventional counterparts. Levels of SAP were similar in all groups tested.

Serum levels of rat SAP and CRP during acute phase responses

The effect of injections of casein or croton oil into male and female rats is shown in Figs 9 and 10. CRP behaved as an acute phase reactant with a maximum rise in concentration of about two-fold. There was no difference between the CRP responses of males and females. SAP levels showed no significant increase in females and only a very minor rise in males.

Functional properties of rat CRP

In marked contrast with human CRP used as a positive control, rat CRP completely failed either to precipitate with CPS or to agglutinate CPS-coated sheep erythrocytes (Tables 4 and 5). 'Native' CRP in fresh rat serum also did not precipitate CPS or agglutinate E-CPS. Addition to rat serum, containing

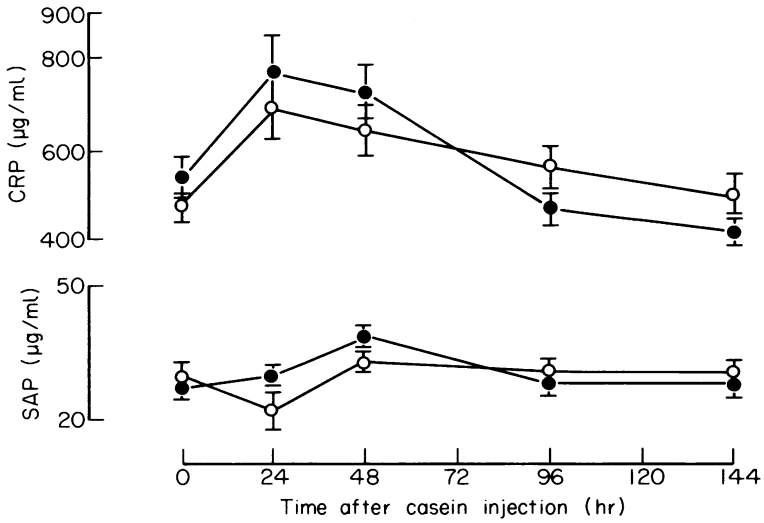

Figure 9. Effect of casein injection on serum levels of CRP and SAP in rats. Each point represents the mean ± SD of five animals in each group. Males (•); females (o).

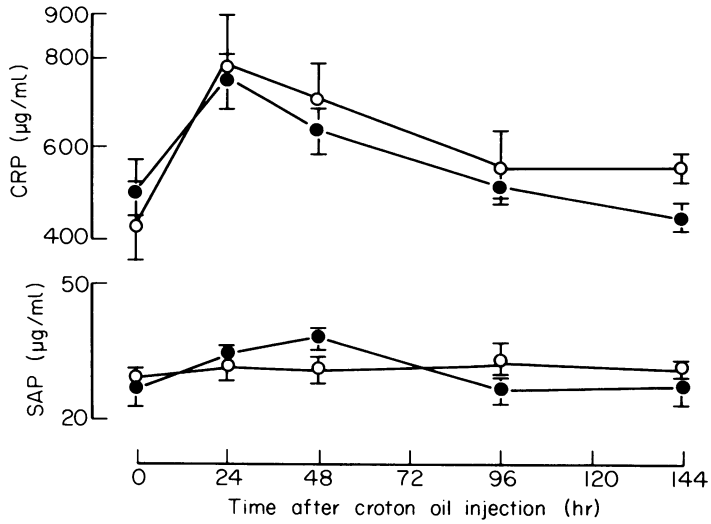

Figure 10. Effect of croton oil injection on serum levels of CRP and SAP in rats. Each point represents the mean ± SD of five animals in each group. Males (•); females (○).

550 μg of endogenous CRP, of amounts of CPS ranging from 0·5–2500 μg/ml failed to activate complement, as measured by C3 conversion. When isolated human CRP (25–625 μg/ml) was added to rat serum together with CPS (15–375 μg/ml) marked C3 conversion occurred, but this was not seen when isolated rat CRP was used (Fig. 11).

DISCUSSION

The identification in rat serum of two proteins with the same calcium-dependent ligand binding specificity as human CRP and SAP, respectively, and with similar appearances in the electron microscope, extends the number of vertebrate species that possess this newly recognized protein family. The amino acid composition of rat CRP is very similar indeed to that of human CRP, and it also has a blocked N-terminal (Oliveira *et*

Table 4. Precipitation of CPS by CRP

CPR:CRP ratio		Precipitation by Human CRP	Rat CRP
CPS excess	64:1	−	−
	32:1	+	−
	16:1	++	−
	8:1	+++	−
	4:1	+++	−
	2:1	+++	−
	1:1	+++	−
CRP excess	2:1	+++	−
	4:1	++	−
	8:1	++	−
	16:1	+	−
	32:1	−	−

Table 5. Agglutination of CPS-coated sheep erythrocytes by CRP in the presence of calcium ions*

Concentration of CRP (μg/ml)	Human CRP	Rat CRP	Rat serum
1000	+	−	ND
500	+	−	−
250	+	−	−
125	+	−	−
63	+	−	−
32	+	−	−
16	+	−	−
8	+	−	−
4	+	−	−
2	+	ND	ND
1	+	ND	ND
0·5	+	ND	ND
0·25	+	ND	ND
0·13	+	ND	ND
0·06	+	ND	ND
0·03	−	ND	ND
None	−	−	−

* Addition of EDTA abolished all agglutination.

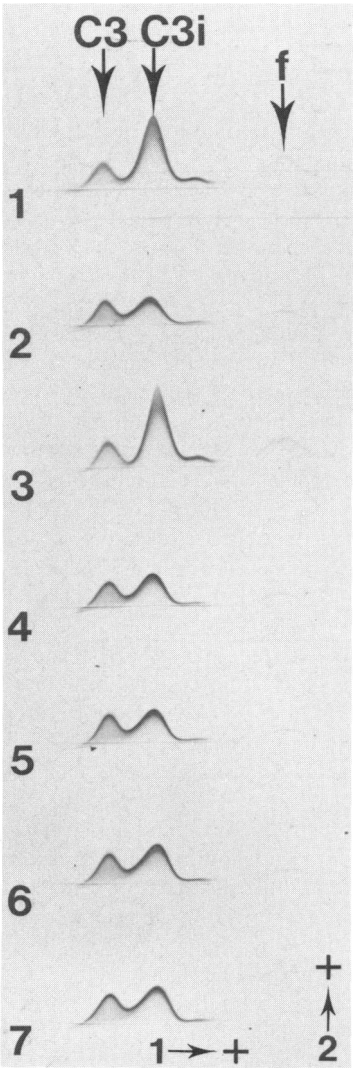

Figure 11. Estimation of C3 conversion in rat serum produced by complexes of rat or human CRP with CPS. Twenty-microlitre volumes of serum had been incubated with the following materials: 1, CoF 0·2 μg; 2, CFT control; 3, human CRP 25 μg + CPS 15 μg; 4, CPS alone 15 μg; 5, human CRP alone 25 μg; 6, rat CRP 25 μg + CPS 15 μg; 7, rat CRP alone 25 μg. The positions of native C3, inactivated C3 (C3i) and a fast mobility split product (f) of C3 are shown by arrows. The directions of electrophoresis in the first and second dimensions are designated 1 and 2, respectively.

al., 1977; 1979), but it differs structurally in being a glycoprotein containing a single complex oligosaccharide unit per subunit whilst human CRP is not glycosylated (Oliveira *et al.*, 1977, 1979; Gotschlich *et al.*, 1965).

In addition rat CRP has some functional properties notably different from those which characterize CRP of other species. Although it binds to CPS, and indeed its isolation depends on that property, we were unable to demonstrate that it precipitates CPS or agglutinates CPS-coated erythrocytes. This is in marked contrast to the behaviour of human, rabbit and plaice CRP all of which are excellent precipitins (Tillet *et al.*, 1930; Anderson & McCarty, 1951; Baldo & Fletcher, 1973). Furthermore neither the endogenous CRP present in fresh rat serum nor isolated rat CRP added into rat serum were able to activate rat complement, either on their own or in the presence of CPS. Complexed human CRP is a potent complement activator (Kaplan & Volanakis, 1974; Siegel, Rent & Gewurz, 1974) and addition of isolated human CRP to rat serum together with CPS caused extensive C3 conversion. Even plaice CRP can activate human complement in the presence of CPS (M.B.P. and T.C. Fletcher, unpublished observations). The capacity of CRP-CPS complexes to activate complement thus seems to be an 'ancient' property of CRP, but one which has been lost by rat CRP at least insofar as autologous complement is concerned. The effect of rat CRP and CPS on complement of other species was not tested.

The differences in the functional properties of rat CRP may be related to its structural configuration. SDS-PAGE of rat CRP under reducing conditions yielded a single main band comprising at least 95% of the protein, but a trace band of slightly higher apparent molecular weight was always present. Both bands were glycosylated. Under non-reducing conditions some subunits ran with a lower apparent molecular weight indicating that they contained intrachain disulphide bridging, but some of the protein ran with an apparently higher molecular weight compatible with interchain disulphide linkage between pairs of subunits. This higher molecular weight band comprised about 40% of the total protein suggesting that there may on average be one such pair of linked subunits per molecule. Evidence of covalent association between subunits within CRP and SAP molecules has not previously been obtained in any other species (Oliveira *et al.*, 1977, 1979; Osmand *et al.*, 1977; Pepys *et al.*, 1978a).

Three separate batches of highly purified rat CRP each gave a single compact, symmetrical peak on elution from Sephacryl S-300 and induced monospecific antisera on repeated immunization of rabbits. One

of these batches was tested by ultracentrifugation and produced linear plots in the meniscus depletion method of sedimentation equilibrium analysis. However all three preparations reproducibly yielded a pattern of four unequal bands in gradient PAGE. Gradient PAGE is a powerful technique for revealing molecular heterogeneity but the basis for these separate bands is not known. It seems unlikely from the present results that they really represent polymorphic forms of CRP with different molecular weights. Furthermore apparent molecular weights of the bands when corrected for the position of human CRP were anomalously low in comparison with the behaviour of rat CRP on gel filtration and in the ultracentrifuge.

Despite the possibly anomalous behaviour of rat CRP and SAP in PAGE techniques, it seems probable from the results obtained, from their behaviour on gel filtration under non-dissociating conditions and from the direct measurement of the molecular weight of rat CRP by sedimentation equilibrium analysis, that both proteins are composed of five rather than ten subunits per molecule. In the case of rat SAP this is supported by its appearances in the electron microscope in which the molecules were seen almost entirely as single pentameric discs with only very occasional pairs interacting face-to-face and no tendency to form stacks. On the other hand a substantial number of pairs of rat CRP pentamers were seen in the electron microscope and there were occasional stacks of such pairs.

There is evidently a gradation across species in the tendency of the basic pentameric annular discs to associate with each other to form two-disc, ten subunit structures and subsequently for these decameric molecules to stack up during preparation for electron microscopy. For example, human CRP is almost always seen as single pentameric discs with only very occasional pairs and no tendency to stack (Osmand *et al.*, 1977; Pepys *et al.*, 1978a), whereas human SAP is always seen as pairs of discs with a variable but always high propensity to form stacks (Osmand *et al.*, 1977; Painter *et al.*, 1976; Pepys *et al.*, 1977a). In the plaice, both SAP and CRP molecules are decameric, contain two discs and make stacks (Pepys *et al.*, 1978a; Pepys *et al.*, 1980). In the mouse, in which only SAP has been studied in sufficient detail, the molecules closely resemble those of human SAP in the electron microscope (E.A.M. and M.B.P., unpublished observations) and run on gel filtration with an apparent mol. wt of 220,000 (Pepys, 1979), but in gradient PAGE they migrate in the same lower molecular weight position as human CRP (Pepys *et al.*, 1978a). This is presumably because under these more extreme physical conditions the two pentameric discs separate from each other, and the rat CRP molecule may also be disrupted by this technique. The formation, in any particular preparation of CRP or SAP, of stable pairs of pentameric discs interacting face-to-face must indicate that, as expected, the two faces of each disc are dissimilar to each other. If the two faces are designated A and B, then in cases where stable pairs are formed there is evidently a relatively strong association between two like faces (say A–A), whilst the appearance of stacks indicates the capacity for a weaker association between the contralateral faces (B–B). This correlates with the observation that when stacks are present, the distance between discs within a pair is less than that between discs of adjacent pairs.

Isoelectric focusing of isolated rat CRP and SAP gave reproducible patterns of closely spaced bands, those of CRP being more anodal (pH 3·8–4·0) than those of SAP (pH 4·1–4·5). The basis for this heterogeneity is not known. Isolated human SAP also gives a series of bands around pH 5·2 whilst isolated human CRP scarcely leaves the cathodal origin in these gels indicating a pI of about 7·9 (M.L.B., F.C. de B. and M.B.P., unpublished observations).

Normal serum levels of SAP were similar in rats to those in man (Pepys, Dash, Markham, Thomas, Williams & Petrie, 1978b), and, as in man, failed to increase significantly during acute phase responses (Pepys *et al.*, 1980). This behaviour differed, however, from that of mouse SAP which is an important acute phase reactant (Pepys, Baltz, Gomer, Davies & Doenhoff, 1979a). On the other hand the levels of rat CRP were remarkably high both in healthy normal conventional animals and in specific pathogen-free rats, compared with the normal level in man of less than 1 μg/ml (Claus, Osmand & Gewurz, 1976; Shine, de Beer & Pepys, 1981). Although they increased further following inflammatory stimuli, the relative increment was modest by comparison with the acute phase response of human CRP (Pepys *et al.*, 1978b; Claus *et al.*, 1976). Young rats had lower serum CRP levels than the adults, although still very high by human standards, suggesting that adult levels may be attributable at least in part to a response to persistent environmental stimuli.

The significance of these differences between SAP and CRP of different species is not clear, particularly as their *in vivo* functions are not known. However, the major differences shown here in structure, functions

and behaviour between rat CRP and CRP of other species suggest new avenues for exploration of the *in vivo* role of CRP and the control of the acute phase response. Rats, unlike mice and men, do not develop amyloidosis (Dunn, 1967; Jakob, 1971; Green, 1974) and it is therefore of interest that rat SAP has a molecular configuration which is different from that of SAP in these amyloid-susceptible species. This finding extends other recent observations which suggest a possible link between P component and the pathogenesis of amyloidosis (Pepys, Dyck, de Beer, Skinner & Cohen, 1979b; Baltz, Gomer, Davies, Evans, Klaus & Pepys, 1980).

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