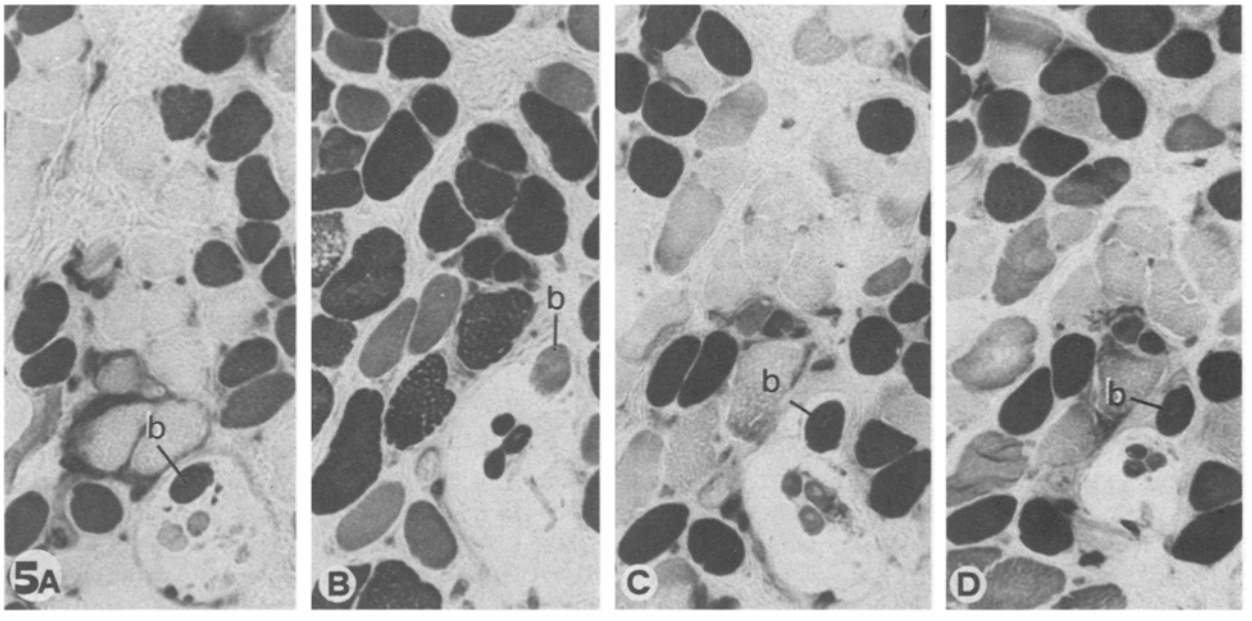

Figure 5. Transverse sections of pig obliquus superior muscle. Note that the spindle presents the bag fiber that moves laterally (*B* and *C*) and leaves the intracapsular space (*D*). *A* m-ATPase, pH 4.5. *B* m-ATPase, after formalin fixation. *C* m-ATPase, pH 4.65. *D* m-ATPase, pH 4.7. b = bag fiber. ×160.

in skeletal muscle spindles, whereas in the pig almost all the spindles contain a single bag fiber and a grouping of chain fibers. Since the unique bag fiber of the pig presents histochemical characteristics which do not completely fit those of bag_1 or bag_2 fibers, further studies will be necessary to better define which type of bag fiber is present in EOM spindles of the pig and its physiological significance.

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Binding property of rat and *Limulus* C-reactive proteins (CRP) to mercury

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Summary. The C-reactive proteins (CRP) from both rat and *Limulus* were found to bind mercury (Hg) in both in vivo and in vitro conditions. CRP has high-affinity binding sites for Hg as evidenced by the loss of free sulfhydryl groups, arrested mobility in polyacrylamide gel electrophoresis, and the consumption of CRP in the serum after Hg administration. The binding was tight as it could not be inhibited either by the addition of cysteine or EDTA. By using a direct titration method it was shown that binding of Hg to CRP was saturable at a molar ratio of Hg/CRP = 13.11. The possibility that CRP may act as a scavenger for Hg is discussed.
Key words. C-reactive protein; mercury; cell necrosis.

C-reactive protein (CRP), discovered in 1930 by Tillett and Francis[1], is one of the acute phase proteins in humans and most animals. In the mammalian system the level of CRP in the serum increases nonspecifically by as much as 1000-fold in response to acute inflammatory processes, tissue necrosis or other pathological conditions[2,3]; however, CRP is also a normal component of the invertebrate haemolymph[4]. CRP is synthesised in the liver, specifically by hepatocytes[5]. Furthermore, CRP has evolved conservatively; homologous proteins with similar functional attributes have been found in many other species. The stable preservation of this protein during evolution implies some biological importance. The finding that CRP is a major blood constituent of primitive animals, e.g. horseshoe crab, *Limulus polyphemus* and dogfish argue strongly for an important role of this protein[6]. It has been reported that rat CRP, a glycoprotein, has a mol.wt of 120,000, and that it consists of five identical subunits, each with a mol.wt of 24,000 and a half-cystine group not involved in any disulphide bridge formation[7]. Electron microscopic studies have revealed that the five subunits of CRP aggregate preferentially as cyclic pentamers and they may stack to form decamers[8]. In its cyclic pentameric form, CRP has a specific CA^{++}-dependent affinity for phosphorylcholine (PC)[9]. According to a recent report chromatin, which is a polyanionic-polycationic structure, also binds specifically to CRP in the presence of calcium ion[6].

In in vitro conditions CRP enhances phagocytosis and inhibits platelet aggregation, mediator release reactions and antigen-induced T-cell activation[10]. In spite of all these functions no definitive role of CRP has yet been established. It is highly probable that a protein which has been in existence for as long as CRP would be polyfunctional[11]. In this report we summarize our findings that CRP has high-affinity binding sites and a very high total binding capacity for mercury, which may lead to detoxication processes to counteract the lethality of this heavy metal.

Materials and methods

Chemicals. Purified *Limulus* CRP, cysteine and agarose (type-IV) were purchased from Sigma Chemical Co. USA. Turpentine was obtained from BH Research Institute, Calcutta, India. Radioisotopic mercury (^{203}Hg) was purchased from Bhabha Atomic Research Centre, Bombay, India. All other chemicals used in this study were of Analar grade and were purchased from Merck/BDH. Double-distilled deionised water was used throughout the experiment.

Animals and treatment. Normal male Sprague-Dawley rats (100 g b. wt), caged in groups of three, which had negative serum CRP values confirmed by double immunodiffusion analysis, were injected with autoclaved turpentine (6 ml · kg^{-1}, i.m.), which was used as an acute phase stimulant for CRP. After 48 h of turpentine treatment, a group of rats was further injected with mercuric chloride ($HgCl_2$, 15 mg · kg^{-1}, i.m.) dissolved in 0.9% NaCl for 24 h. Water and food were available ad libitum. Blood was collected directly by cardiac puncture of anaesthetized rats, and the serum separated by centrifugation (100 × g; 30 min) and stored at −20 °C until required. All the sera were tested by double immunodiffusion analysis against the antiserum to *Limulus* CRP raised in rabbit.

Urine collection. Urine was collected directly from the urinary bladder. It was then tested for the presence of CRP by double immunodiffusion.

Preparation of antiserum. Rabbit antiserum[4] to *Limulus* CRP was raised by immunization of a white male rabbit. An emulsified solution of *Limulus* CRP (0.6 mg; 1 mg/ml) and Freund's Complete Adjuvant (0.6 ml) was injected s.c. into the rabbit. This was followed by two booster injections of the same amount one week and three weeks later. Six weeks after initial injection the rabbits were bled by cardiac puncture and antiserum prepared from the clotted whole blood. The specificity of the antiserum was tested against purified *Limulus* CRP and rat CRP stimulated by turpentine, by double immunodiffusion. The antiserum was stored at −20 °C until required.

Double immunodiffusion studies. Immunodiffusion studies were done on 1% agarose-covered microscope slides in 75 mM barbital buffer (pH 8.6) containing 100 mM NaCl and 10 mM EDTA. About 100 µl of crude serum was loaded into the holes made into agarose gel and the slides were left at room temperature for 48 h.

Direct titration assay. Various concentrations of radioisotopic mercury (^{203}Hg) were incubated at room temperature for 1 h with 50 µg of purified *Limulus* CRP (1 mg/ml in 10 mM phosphate buffer pH 7.4). The amount of protein was held constant (100 pmoles) while the total amount of ^{203}Hg was varied from 5 pmoles to 5000 pmoles (5×10^{-9} to 5×10^{-6} moles/l). The final volume was made up to 1.0 ml. Free and CRP bound ^{203}Hg were separated by polyethylene glycol (10%) precipitation and the radioactivity was quantitated by Geiger-Müller Counter (ECIL, India). ^{203}Hg was added to CRP until the added ^{203}Hg remained free in solution. A molecular weight of 5×10^5 daltons for *Limulus* CRP was used to calculate the molar concentration of the protein.

In vitro studies. Purified *Limulus* CRP (100 µg) was incubated at room temperature for 1 h with 1 mM $HgCl_2$ and 75 mM barbital buffer (pH 8.6), giving a final volume of 1.0 ml. Free unbound mercury was separated from bound CRP by PEG precipitation as described earlier. Immediately after incubation an aliquot of it was used for electrophoretic analysis and convenient aliquots were taken to check the mobility of CRP on agarose plates. Free sulfhydryl groups were also estimated in the incubation mixture according to the method of Ellman[12].

Polyacrylamide gel electrophoresis (PAGE). Electrophoretic mobility of CRP from whole crude serum, purified *Limulus* CRP and the incubation mixture of *Limulus*

CRP and Hg was checked in both 5% and 7.5% polyacrylamide gel according to the method of Davis [13]. A control with 1 mM $HgCl_2$ alone was also run in a gel under identical conditions. Each gel was run in triplicate. One set of gels was stained with Coomassie Blue for general proteins. Another set was kept in antiserum against *Limulus* CRP for 48 h for specific protein precipitation and the third gel was dipped in yellow ammonium sulphide $(NH_4)_2S$ solution to detect the presence of Hg.

Estimation of phospholipid. Phospholipids from the serum were extracted and their phosphate content was measured according to the method of Fiske and Subbarow [14].

Results

On Ouchterlony slides, purified *Limulus* CRP and serum from turpentine-treated rat were found to produce a single precipitin line against the antiserum while the serum from untreated rat and saline-treated rat did not demonstrate such a reaction. To our surprise the serum from the rats treated with turpentine followed by $HgCl_2$ injection did not produce a precipitin line; instead, a white ring appeared around the well on immunodiffusion plate. This ring was similar to the ring produced by the serum from rats treated only with mercury. Although the serum from Hg-treated rats did not produce a precipitin line on agarose slides, Hg was found to cause a rise in the level of CRP in serum, as evidenced by test-tube immunoprecipitation technique (unpubl. obs.). Administration of 0.9% NaCl into the rats after stimulation of CRP by turpentine did not interfere with the level of CRP in serum. Urine collected either from the turpentine-treated or from the mercury-treated rats did not give a precipitin band against the antiserum. Presence of CRP either in the serum or in the urine could not be detected in the rat receiving both turpentine and mercury injections. None of the samples reacted with normal rabbit serum. The incubation mixture of purified *Limulus* CRP and $HgCl_2$ produced a precipitin line against antiserum identical to the precipitin line obtained with only CRP. $HgCl_2$ alone did not affect the mobility of *Limulus* CRP on an immunodiffusion plate (fig. 1; table 1).

The direct titration of *Limulus* CRP and ^{203}Hg suggested that CRP was binding to Hg. Data indicated that maximum binding (total binding capacity) of ^{203}Hg to

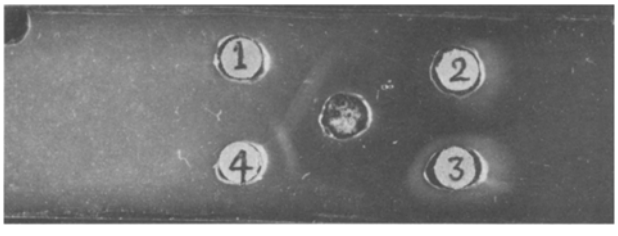

Figure 1. Double immunodiffusion pattern done in 1% agarose gel. Well 1 contains *Limulus* CRP. Well 2 contains serum from rat treated with turpentine followed by mercury injection. Well 3 contains serum from mercury-treated rat. Well 4 contains serum from turpentine-treated rat. Center well contains antiserum to *Limulus* CRP.

Table 1. CRP in the serum and urine of normal and treated rats detected by double immunodiffusion tests

Treatment	Duration (h)	CRP Serum	Urine
1 Normal untreated rat	–	Negative	Negative
2 Turpentine-treated rat	48	Positive	Negative
3 $HgCl_2$-treated rat	24	Positive*	Negative
4 0.9 NaCl-treated rat	24, 48	Negative	Negative
5 Turpentine-treated rat injected with $HgCl_2$	48 + 24	Positive*	Negative
6 Turpentine-treated rat injected with 0.9% NaCl	48 + 24	Positive	Negative

*Confirmed by test-tube immunoprecipitation technique.

Table 2. Binding of Hg at sulfhydryl groups of CRP and cysteine

System	Free sulfhydryl groups (absorbance at 412 nm)
CRP (100 µg)	0.678
CRP (100 µg) + $HgCl_2$ (1 mM)	0.000
$HgCl_2$ (1 mM)	0.000
Cysteine (4 mM)	1.234
Cysteine (4 mM) + $HgCl_2$ (1 mM)	0.000

50 µg of CRP was obtained at a concentration of 13.11×10^{-7}M Hg. The molar ratio of Hg/CRP = 13.11 was calculated from the binding of 1311 pmoles of Hg to 100 pmoles of CRP at saturation level. This suggests the presence of multiple binding sites in CRP for Hg; however, it is not possible to say whether these sites are equivalent. Addition of EDTA or cysteine in the incubation mixture could not dislodge the bound Hg from CRP. Direct proof of such an interaction between Hg and CRP was obtained by assaying the sulfhydryl group and performing polyacrylamide gel electrophoresis. Purified *Limulus* CRP showed the presence of free sulfhydryl groups, while in the incubation mixture containing CRP and $HgCl_2$ no free sulfhydryl groups could be detected. Likewise, the free sulfhydryl groups of cysteine were blocked by $HgCl_2$ (table 2).

Whole serum from the mercury-treated rat failed to migrate on polyacrylamide gel with respect to CRP, as demonstrated by a white precipitate on the top of the gel when it was kept in antiserum for 48 h. The top end of a replicate gel gave a positive reaction with Coomassie Blue. However, in $(NH_4)_2S$ solution it did not reveal the presence of mercury. Purified CRP gave a single band with Coomassie blue and a clear precipitin band in antiserum after treatment for 48 h at room temperature. Purified CRP, when incubated with $HgCl_2$, also failed to migrate either in 5% or 7.5% polyacrylamide gel. Furthermore, as noted with the serum of mercury-treated rats, when this gel was kept in antiserum, the top cathodal end showed a white precipitate. In $(NH_4)_2S$ solution it turned black showing the presence of Hg (fig. 2). The mobility of 1 mM solution of $HgCl_2$ was checked in both 5% and 7.5% polyacrylamide gels. In none of the cases the top of the gel turned black on treatment with $(NH_4)_2S$. The electrophoretic mobility could not be recovered either by adding 10 mM EDTA or 4 mM cysteine in the electrophoresis buffer.

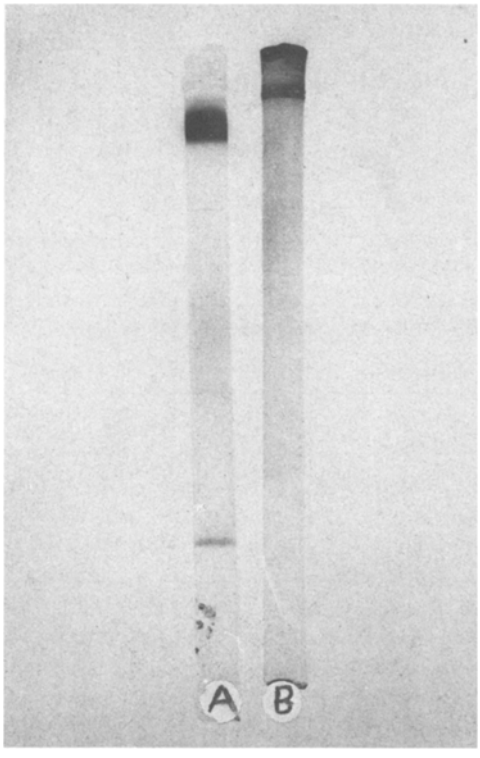

Figure 2. 7.5% PAGE of *Limulus* CRP (gel A) and *Limulus* CRP + $HgCl_2$ (gel B) run under nondissociating conditions.

Discussion. The rats injected with turpentine had an elevated level of CRP, and this CRP could not be detected by double immunodiffusion after Hg-treatment either in the serum or in urine from the same rat. This indicates that CRP has not been excreted, but it may have been consumed in the serum itself. CRP has been reported to have a unique property of binding to phosphoryl choline (PC) attached to a protein [15] or to carbohydrates [9] in the presence of calcium. After binding with mercury, as evidenced by the serum of the mercury-treated rat, CRP could not be detected by immunodiffusion tests. Instead, CRP reacted with the agarose, producing a white ring around the well. A weak Ca^{++}-dependent reaction between CRP and agarose has been reported earlier [16]. We suspect that a complex of Hg and PC-containing necrotic products caused by Hg itself is responsible for the binding of CRP to agarose, because purified *Limulus* CRP incubated with $HgCl_2$ gave clear precipitin lines on agarose plates. The in vivo binding of CRP to necrotic cells has already been identified [10]. There is no information regarding the mode of disappearance of CRP from the serum. In the present investigation, EDTA and cysteine were not able to chelate Hg from CRP. This suggests that a complex formation between CRP, Hg and necrotic products has taken place. Injection of Hg either in normal rats or in turpentine-treated rats showed an elevated level of phospholipid (about three times higher; data not shown) in their serum, which is suggestive of tissue damage.

The findings obtained with purified *Limulus* CRP, showing the absence of free sulfhydryl groups after addition of $HgCl_2$, substantiates our observations with in vivo experiments. It proves that CRP binds Hg and this binding occurred at the free sulfhydryl groups of cysteine on CRP. Analysis of the binding affinity of CRP towards Hg by direct titration suggests the existence of multiple ligand binding sites. Since each subunit of CRP has only half-cystine group, a molar ratio of Hg/CRP = 13.11 is plausible only when other sites take part in Hg-binding. The presence of multiple ligand-binding sites and a very high total binding capacity of CRP for Hg also reflects the possible role of CRP in sequestration of heavy metals. The electrophoretic mobility of CRP either from Hg-treated crude serum or purified CRP + $HgCl_2$ was found to be lost and the cathodal end of the gel showed the presence of both CRP and Hg, which indicates that CRP and Hg are bound together. The retardation in the electrophoretic mobility of mammalian CRP in the presence of calcium has already been shown [17]. Comparison between rat CRP and purified *Limulus* CRP indicates that there is a cross-reaction between the two, and that they are remarkably similar in their behaviour towards mercury.

It has been hypothesized that the main role of CRP, for which it evolved and has been conserved, is to recognize in the plasma potentially toxic autogenous materials released from damaged tissues, to bind to them and thereby to detoxify them and/or facilitate their clearance [10]. From our observations on CRP in relation to mercury, it may be concluded that in addition to the aforementioned role of CRP in acute phase response of animals it may have another important role in detoxication processes, and thus CRP may be redefined as a polyfunctional acute phase protein.

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