A new haemagglutinin from the amoebocytes of the horseshoe crab *Carcinoscorpius rotundicauda*

Purification and role in cellular aggregation

Subita SRIMAL, D. Thambi DORAI, Mohan SOMASUNDARAN, Bimal Kumar BACHHAWAT*
and Toshiyoki MIYATA†

Indian Institute of Chemical Biology, 4 Raja Subodh Mullick Road, Jadavpur, Calcutta 700 032, India

(Received 2 January 1985/20 March 1985; accepted 15 May 1985)

The present paper describes the purification and function of a haemagglutinin from the amoebocyte lysate of the horseshoe crab *Carcinoscorpius rotundicauda*. The purified protein consisted of a single subunit of M_r 24000 and agglutinated human blood-group-A⁺ erythrocytes. Its haemagglutinin activity was inhibited by purified lysate, coagulogen, but not by sugars. The haemagglutinin differed immunologically and in activity from the sialic-acid-binding lectin carcinoscorpin present in the haemolymph. It caused aggregation of formalin-fixed amoebocytes, and on the basis of this observation its role in cell–cell adhesion is proposed. This new haemagglutinin promotes cell–cell aggregation in amoebocytes in a manner that shares some similarities with thrombospondin-mediated platelet aggregation in vertebrates [Jaffe, Leuang, Nachman, Levin & Moseher (1981) *Nature (London)* **295**, 246–248].

Noguchi (1903) first detected haemagglutinin activity in the haemolymph of the horseshoe crab *Limulus*, and later similar substances were found to occur in the haemolymph and tissue extracts of many invertebrate species (Yeaton, 1981a). Several haemagglutinins from different species of the horseshoe crab have been well characterized (Marchalonis & Edelman, 1968; Bishayee & Dorai, 1980; Shishikura & Sekiguchi, 1983; Dorai *et al.*, 1982a,b), but their sites of synthesis and function in the animal remain unknown. In invertebrates the haemocytes/amoebocytes that play an important role in haemostasis and the host-defence mechanism have been implied as the site of lectin synthesis (Yeaton, 1981b). Aggregation and adhesion of these cells is a prerequisite for haemostasis, but the mechanism underlying cell aggregation is not known (Levin, 1967). Diverse functions have been assigned to invertebrate lectins, including their role in mediating cellular

aggregation (Yeaton, 1981b). Previous studies in our laboratory have demonstrated the presence of a haemagglutinin called 'carcinoscorpin', in the haemolymph of the horseshoe crab *Carcinoscorpius rotundicauda*. In the present paper we report the presence of a new haemagglutinin within the amoebocyte of *C. rotundicauda* that is different from carcinoscorpin.

Materials and methods

Sialic acid, disialylsaccharide, 2-oxo-3-deoxy-octonate, D-glucuronic acid, D- and L-fucose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylmannosamine, mannosamine, D-galactose, D-glucose, L-glucosamine, colomonic acid, heparin, phosphocholine, β -glycerophosphate, arginine, lysine, histidine, gelatin, fetuin (type IV), LPS (from *Salmonella typhimurium*), Triton X-100, sodium deoxycholate (DOC), benzamidinium hydrochloride and di-isopropyl fluorophosphate (DFP) were purchased from Sigma. DEAE-Sephadex A-50 and Sephadex G-50 (medium grade) were purchased from Pharmacia. Heparin-Sepharose CL-6B was prepared by the method of March *et al.* (1974). All other reagents used were of analytical grade. Pyrogen-free glassware and reagents were used for the experiments.

Abbreviations used: SDS, sodium dodecyl sulphate; HA, haemagglutinin activity; β -Me, β -mercaptoethanol; LPS, lipopolysaccharide; DFP, di-isopropyl fluorophosphate; DOC, deoxycholate.

* To whom correspondence and reprint requests should be sent.

† Present address: Department of Biology, Faculty of Science, Kyushu University, 33, Fukuoka, 812, Japan.

Preparation of amoebocyte lysate

Adult horseshoe crabs (*C. rotundicauda*) were bled by cardiac puncture and the amoebocyte lysate prepared (Jorgenson & Smith, 1973). The amoebocyte lysate was incubated at 37°C for 1 h with 1 µg of LPS (*S. typhimurium*) and the clot formed was removed by centrifugation. The supernatant left after removal of the clotted protein was assayed for haemagglutination.

Haemagglutination assay

A 2% erythrocyte suspension was made from heparinized blood of sheep, horse, rabbit and of the human A⁺ or O⁺ group. The washed erythrocytes were suspended in 50 mM-Tris/HCl, pH 8.0, containing 100 mM-NaCl and 100 mM-CaCl₂ (Bishayee & Dorai, 1980). The Ca²⁺ requirement was tested in iso-osmotic buffer lacking CaCl₂. Haemagglutination was carried out in microtitre plates. A 50 µl portion of the test material was serially diluted (in twofold series) in the buffer, followed by the addition of 50 µl of the suspension of erythrocyte of different species. Haemagglutinating titre was recorded after incubation for 1 h at room temperature. Specific activity is defined as titre/mg of protein. Inhibition of erythrocyte agglutination with different amino acids, sugars or proteins was recorded after incubating the sample with various amounts of the inhibitors for 30 min at room temperature, followed by the addition of human blood-group-A erythrocytes. The sialic-acid-binding lectin concanavalin (Bishayee & Dorai, 1980) was used to raise antibody in rabbits. Immunodiffusion was carried out by using 1% agar containing 0.02% NaN₃ and 20 mM-citrate (citrate was used to inhibit the lectin-ligand interaction of concanavalin).

Purification of haemagglutinin from the amoebocyte lysate

Amoebocytes from 150 ml of haemolymph (collected under pyrogen-free conditions) were lysed by freezing and thawing. The lysed cells were suspended in 30 ml of pyrogen-free saline containing 2 mM-DFP, 10 mM-benzamidine hydrochloride and 0.02% NaN₃ and homogenized in a Physcotron (Nihon-Seimitsu Kogyo Ltd., Tokyo, Japan) homogenizer for 2 min at 0°C. The homogenate was centrifuged at 10000g for 30 min at 4°C in a Hitachi 20 PR-52 centrifuge and the supernatant (lysate) used for purification of the haemagglutinin.

Gel filtration on Sephadex G-50

A 30 ml portion of the lysate (4.3 mg of protein/ml) was applied to a column (3 cm × 140 cm) of Sephadex G-50 equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-benzamidine

hydrochloride, 0.02% NaN₃ and 0.1 M-NaCl. The protein elution profile was monitored by measuring A₂₈₀ and the fractions were assayed for HA and analysed by SDS/polyacrylamide-gel electrophoresis. Fractions containing HA were pooled and were dialysed against 50 mM-Tris/HCl, pH 7.5, containing 10 mM-benzamidine hydrochloride and 0.02% NaN₃. The dialysed protein solution was applied to a column (3 cm × 27 cm) of heparin-Sepharose equilibrated with the above buffer. The breakthrough fractions and proteins eluted by a linear NaCl gradient were precipitated by 40% (w/v, final concn.) (NH₄)₂SO₄ separately. The precipitate was dissolved in 0.05 M-ammonium bicarbonate buffer, pH 7.8, dialysed extensively against the same buffer, assayed for HA and analysed by SDS/polyacrylamide-gel electrophoresis. The purified haemagglutinin was freeze-dried and stored at -20°C.

Immunoprecipitation of haemagglutinin from the whole lysate

To prepare anti-haemagglutinin antibody, adult male albino rabbits were immunized subcutaneously at multiple sites on the back with 250 µg of electrophoretically pure haemagglutinin in Freund's complete adjuvant. Booster doses (125 µg of protein) were given after 3 and 4 weeks with incomplete Freund's adjuvant. The rabbits were bled after 4 weeks and the antisera separated and stored at -20°C. Pre-immune serum was collected before immunization.

Portions of amoebocyte lysate (4.3 mg of protein/ml) were incubated at 4°C overnight with 300 µl of antisera in a final volume of 1 ml in the presence of 10 mM-benzamidine hydrochloride and 0.02% NaN₃. In control experiments pre-immune serum was used. The immunoprecipitate obtained was separated by centrifugation in a Microfuge at full speed for 3 min and washed twice with solution containing 1% Triton X-100, 0.5% DOC and 0.15 M-NaCl by the method of Cladaras & Cottam (1980). The immunoprecipitate was finally dissolved in Laemmli's (1970) SDS/polyacrylamide-gel electrophoresis buffer containing β-Me and analysed by SDS/polyacrylamide-gel electrophoresis.

Studies on the interaction between coagulogen and haemagglutinin

Various amounts of coagulogen (2 mg/ml) were added to tubes containing 250 µg of purified haemagglutinin (A₂₈₀ = 0.280) in a total volume of 1 ml of buffer containing 50 mM-Tris/HCl, pH 8.0, and 0.15 M-NaCl. After 4 h incubation at 4°C the u.v.-absorption spectra (220–340 nm) of pure coagulogen, haemagglutinin and assay tubes were recorded with a Hitachi model-220 A spectro-

photometer at a scan speed of 10 nm/cm and with a full scale of 0–0.5 A.

Amino acid analysis

Amino acid analysis was performed on the electrophoretically pure protein after hydrolysis with 6M-HCl for 24, 48 and 72 h and with 4M-methanesulphonic acid for 24 h at 110°C. After evaporation the hydrolysates were analysed on a Hitachi 835 automatic analyser.

Electrophoresis

The proteins were analysed by polyacrylamide-gel electrophoresis in the presence of SDS. SDS/polyacrylamide-gel electrophoresis was performed on a 12.5%-(w/v)-polyacrylamide slab gel (0.1 cm × 10 cm × 12 cm) with a 5% stacking gel (Laemmli, 1970). The proteins were stained with Coomassie Blue R-250. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Aggregation of the amoebocytes

Amoebocytes from 100 ml of haemolymph were fixed in 3% (w/v) NaCl containing 20 mM neutralized formaldehyde (Rickles *et al.*, 1979). The fixed cells after extensive washing were examined for integrity under a phase-contrast microscope and used for subsequent experiments.

Aggregation of the amoebocytes by the purified haemagglutinin was observed by phase-contrast microscopy and also by measuring the decrease in A_{510} . To 1 ml of the cell suspension ($A_{510} = 0.36$ /ml) 50 µg of the haemagglutinin was added and the decrease in absorbance with time was measured at 510 nm.

Results

Amoebocyte lysate haemagglutinated erythrocytes of various species in the following order: human blood-group A > horse > sheep > human blood-group O > rabbit. The specific activity is given in Table 1. In control experiments LPS or purified coagulogen did not have HA. The haemagglutinin exhibited species-specificity and required Ca^{2+} for its activity. Despite this Ca^{2+} -ion requirement, EDTA (100 mM) did not inhibit the haemagglutination.

The purification of the haemagglutinin from the amoebocyte lysate was performed in the presence of proteinase inhibitors. During gel filtration on Sephadex G-50 the haemagglutinin was eluted out in the second peak (II, Fig. 1). However, the HA peak did not coincide with the protein peak (I). SDS/polyacrylamide-gel electrophoresis analysis of the various fractions is shown in Fig. 2. Fraction 55, containing the highest HA (Fig. 2, lane f) was

Table 1. Agglutination of erythrocytes from various species by the amoebocyte lysate of the horseshoe crab *C. rotundicauda*

Erythrocytes	Specific activity (titre/mg of protein)
Human	
Blood-group A	
+ Ca^{2+}	413
– Ca^{2+}	50
Blood-group O	25
Horse	102
Sheep	51
Rabbit	8

Fig. 1. Molecular-sieve chromatography of whole lysate. Amoebocyte lysate (130 mg of protein/30 ml) containing 2 mM-DFP, 10 mM-benzamidine hydrochloride, 0.02% NaN_3 and 2 mM-EDTA in 50 mM-Tris/HCl buffer, pH 7.5, was applied on to a column (3 cm × 140 cm) of Sephadex G-50 equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-benzamidine hydrochloride, 0.02% NaN_3 and 100 mM-NaCl, at a flow rate of 40 ml/h. The protein elution profile was monitored at A_{280} and the HA assayed, as described in Materials and methods section, with human blood-group-A⁺ erythrocytes.

found to be relatively free of coagulogen, in contrast with fractions 49–51 (Fig. 2, lanes b–e). By heparin–Sephacrose chromatography the haemagglutinin could be separated from coagulogen (Fig. 3); a 35-fold purification was achieved and approx. 4.5 mg of pure haemagglutinin (having a specific activity of 81920 HA units/mg of protein) was obtained from 150 ml of haemolymph. The

Fig. 2. Analysis of proteins in fractions obtained from gel filtration of amoebocyte lysate (Fig. 1) by SDS/12.5%-polyacrylamide-gel electrophoresis

Aliquots (50 μ l) from each fraction were treated with Laemmli's sample buffer containing β -Me and applied in separate lanes. (a) Fraction no. 34; (b) fraction no. 49; (c) fraction no. 50; (d) fraction no. 51; (e) fraction no. 52; (f) fraction no. 55; (g) M_r markers: phosphorylase *b* (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), Soya-bean trypsin inhibitor (20100) and α -lactalbumin (14000).

Fig. 3. Heparin-Sepharose chromatography

Further purification of the haemagglutinin was achieved by heparin-Sepharose chromatography. Fractions possessing HA after Sephadex G-50 gel filtration of amoebocyte lysate were pooled, dialysed and applied to a heparin-Sepharose column (3 cm $\times$ 27 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-benzamidine hydrochloride and 0.02% NaN_3 . The bound proteins were eluted by a linear NaCl gradient (0–0.3 M) and the HA was assayed. Peak I contains haemagglutinin.

Fig. 4. SDS/12.5%-polyacrylamide-gel electrophoresis of haemagglutinin under reducing and non-reducing conditions (a) In the absence of β -Me; (b) M_r markers (same as for Fig. 2); (c) in the presence of β -Me. Approx. 10 μ g of protein was applied in each lane.

heparin-Sepharose purification step was accompanied by an increase in the total activity, suggesting the removal of inhibitor(s). On SDS/polyacrylamide-gel electrophoresis under reducing and non-reducing conditions the haemagglutinin was found to be homogeneous and comprised a single subunit of M_r 24000 (Fig. 4). The haemagglutinin did not hydrolyse coagulogen or the synthetic chromogenic peptide benzyloxy-carbonyl-Leu-Gly-Arg *p*-nitroanilide.

Antibody raised against the haemagglutinin gave a single immunoprecipitin line and did not cross-react with coagulogen or any other haemolymph proteins. SDS/polyacrylamide-gel electrophoresis of the immunoprecipitate obtained from the whole lysate in the presence of proteinase inhibitors (Fig. 5) revealed that, in the crude lysate also, the haemagglutinin existed as a protein with a M_r of 24000 and was not modified during the isolation steps.

The amino acid composition of the haemagglutinin is given in Table 2. It has a high content of phenylalanine, tyrosine, leucine, isoleucine and acidic amino acids and contains about 0.9% glucosamine. Its M_r , as calculated from the amino-acid-analysis data, is 24640, which correlates with that determined by SDS/polyacrylamide-gel electrophoresis (M_r 24000).

Interaction of haemagglutinin with coagulogen

Gel filtration of the amoebocyte lysate was carried out on a Sephadex G-50 column (Fig. 1), and the u.v. spectra of the various fractions are shown in Fig. 6(a). In spectrum 3 (fraction no. 55) the shoulder at 288 nm is distinct, whereas in spectra 1 and 2 (fractions no. 49 and 50) a

Fig. 5. Immunoprecipitation of amoebocyte lysate proteins by haemagglutinin antisera

Antisera (300 μ l) was incubated with various amounts of lysate (400 mg/ml) in the presence of 10 mM-benzamidine hydrochloride and 0.02% NaN_3 overnight at 4°C. The immunoprecipitate was separated and washed by the method described in the Materials and methods section and analysed by SDS/12.5%-polyacrylamide-gel electrophoresis. (a) Whole-cell lysate (160 μ g of protein); (b) control (precipitate obtained on incubation of 300 μ l of whole-cell lysate with 300 μ l of pre-immune sera). Precipitate obtained after incubation of 300 ml of HA antisera with (c) 100 μ l of lysate, (d) 200 μ l of lysate, (e) 300 μ l of lysate; (f) M_r markers (same as for Fig. 2). H, IgG heavy chain; L, IgG light chain; C, coagulogen; HA, haemagglutinin.

Table 2. Amino acid composition of haemagglutinin isolated from *C. rotundicauda* haemocytes

Amino acid	Composition (residues per molecule)
Asp	24.9 (25)*
Thr	11.2 (11)
Ser	18.8† (19)
Glu	18.1 (18)
Pro	12.2 (12)
Gly	24.7 (25)
Ala	14.2 (14)
Cys	8.4† (8)
Val	8.7† (9)
Met	2.6 (3)
Ile	10.7† (11)
Leu	15.9† (16)
Tyr	12.7 (13)
Phe	14.3 (14)
Lys	11.2 (11)
His	2.9 (3)
Trp	4.4‡ (4)
Arg	8.1 (8)
Total	224
GlcN	2.0

* Values in parentheses are to nearest integer.

† Calculated from values determined for 24, 48 and 72 h hydrolysis back-extrapolated to zero time.

‡ Determined by the methanesulphonic acid method.

flattening of the shoulder is observed. The haemagglutinin is relatively free of coagulogen in fraction no. 55 (Fig. 2, lane f). Thus experiments were further designed to study the interaction between the purified haemagglutinin and coagulogen. The u.v. spectra (220–340 nm) of purified haemagglutinin, coagulogen and their mixture are shown in Fig. 6(b). A shoulder at 288 nm typical of tryptophan is seen in spectrum no. 2 (Fig. 6b), which is absent from spectrum no. 1 of coagulogen. Addition of coagulogen to the purified haemagglutinin resulted in a gradual flattening of the 288 nm shoulder (spectra 3, 4, 5 and 6 in Fig. 6b), indicative of a protein–protein interaction.

The HA of the crude and purified protein was not inhibited by the sugars known to inhibit carcinoscorpin, i.e. sialic acid (100 mM), 2-oxo-3-deoxyoctonate (100 mM), glucuronic acid (200 mM), disialylsaccharide (100 mM), β -glycerophosphate and phosphocholine (10 mM), and other sugars such as *N*-acetylglucosamine. *N*-Acetylgalactosamine, mannosamine, glucosamine, glucose, galactose, fucose and heparin also did not inhibit the HA. Of the several proteins tested, only fetuin, asialofetuin (3 mg/ml) and coagulogen (0.35 mg/ml) inhibited haemagglutination (100% inhibition). Amino acids (lysine, arginine and histidine), and proteins such as bovine serum albumin and ovalbumin, were ineffective. The purified protein did not cross-react with antisera raised against carcinoscorpin. Immunoprecipitation and immunofluorescence studies also indicated that carcinoscorpin is not present in the amoebocytes (results not shown). Aggregation of the amoebocytes by the purified haemagglutinin and the partially purified protein was measured spectrophotometrically (Fig. 7). When observed under the microscope, aggregation was found to be associated with an alteration in the cell shape (Fig. 8).

Discussion

The haemagglutinin purified by the above method yields an active protein that also appears to be a major lysate protein like coagulogen. That it is not modified by the proteinases of the clotting system is evidenced by the immunoprecipitation studies, where no other lysate protein or a higher- M_r haemagglutinin is precipitated. The apparent association of coagulogen and haemagglutinin in the lysate is indirectly suggested by careful analysis of the Sephadex G-50 gel-filtration profile of the lysate (Fig. 1). The elution of the probable haemagglutinin–coagulogen complex (fractions 48–51) is followed by that of an unassociated haemagglutinin (fraction 55). This is significant, since coagulogen, despite its smaller size (M_r

Fig. 6. (a) U.v.-absorption spectrum of proteins in fractions obtained on Sephadex G-50 gel filtration of whole lysate and (b) interaction of purified haemagglutinin with purified coagulogen

(a) Fractions from Peak II of Fig. 1 were scanned at a chart speed of 10nm/cm in the u.v. range 220–340nm. 1, Fraction no. 49; 2, fraction no. 50; 3, fraction no. 55. (b) The u.v. spectrum of purified (1) coagulogen (400 μ g/ml), (2) haemagglutinin (280 μ g/ml), (3) haemagglutinin (250 μ g) and coagulogen (100 μ g), (4) haemagglutinin (250 μ g) and coagulogen (200 μ g), (5) haemagglutinin (250 μ g) and coagulogen (2300 μ g) and (6) haemagglutinin (250 μ g) and coagulogen (400 μ g). The final volume was 1 ml and all protein solutions were made in

20000) and cationic nature, was eluted before the higher- M_r (24000) protein. The specific activity of the haemagglutinin is higher in fractions relatively free of coagulogen, implying modulating/inhibitory activity of coagulogen on the HA. Removal of coagulogen by heparin-Sepharose chromatography is accompanied with an increase in total activity as well as a 34-fold purification. The yield, calculated on the basis of the HA, is higher than 100% because inhibitors are removed during the purification steps. The haemagglutination was not inhibited by the above-mentioned sugars, but was inhibited by coagulogen and fetuin, suggesting that, *in vivo*, a protein-protein type of interaction occurs. Addition of purified coagulogen to the pure haemagglutinin results in a change in the u.v. spectra of the protein, suggesting the involvement of the haemagglutinin tryptophan residues in the interaction with coagulogen. Aggregation of the amoebocytes in the presence of the haemagglutinin suggests the presence of its ligand on the cell surface.

Fig. 7. Aggregation of formalin-fixed amoebocytes by the haemagglutinin

The aggregation was measured by observing the decrease in A_{510} with time. A 1 ml portion of the cell suspension ($A_{510} = 0.36$ /ml) in 50mM-Tris/HCl, pH 8.0, containing 100mM-NaCl was used. ●, Control (without haemagglutinin); ○, after addition of partially purified haemagglutinin (50 μ g); △, after addition of purified haemagglutinin (50 μ g).

50mM-Tris/HCl buffer, pH 8.0, containing 100mM-NaCl. Scans were recorded in the u.v. range 220–340nm at a chart speed of 10nm/cm; full scale was 0–0.5A.

Fig. 8. Agglutination of the amoebocytes by the haemagglutinin as observed under phase-contrast microscope. Samples from the assay tubes of Fig. 7 were taken and observed. (a) Control, without haemagglutinin (magnification $\times 80$); (b) agglutinated cells after addition of haemagglutinin (magnification $\times 80$); (c) agglutinated cells after addition of haemagglutinin (magnification $\times 310$).

In connection with the above findings, attention is drawn to a thrombin-sensitive protein (thrombospondin), isolated from platelets, which is known to possess HA (Jaffe *et al.*, 1981). Recent studies have shown that both thrombospondin and fibrinogen are secreted from the α -granules of the platelets and become integrated into the plasma

membranes (Gardner *et al.*, 1980). Thrombospondin mediates platelet aggregation, and the aggregation is inhibited by fibrinogen. On the basis of these observations it is postulated that fibrinogen is the receptor for platelet lectin (thrombospondin), which mediates cell aggregation (Gardner *et al.*, 1981). Our studies also show that the amoebocytes are aggregated by the haemagglutinin and the haemagglutination is inhibited by coagulogen. Hence we propose that aggregation in the amoebocytes is mediated by the haemagglutinin and that coagulogen is its receptor.

We thank Professor S. Iwanaga, Department of Biology, Kyushu University, Fukuoka, Japan, for his suggestions, and Mrs. K. Kawaguchi for the amino acid analysis. We thank Dr. Sandip K. Basu for his help in the preparation of the manuscript. This work was supported in part by grants from the Department of Science and Technology, Government of India.

References

- Bishayee, S. & Dorai, D. T. (1980) *Biochem. Biophys. Acta* **623**, 89–97
- Cladaras, C. & Cottam, G. L. (1980) *J. Biol. Chem.* **255**, 11499–11503
- Dorai, D. T., Srimal, S., Mohan, S., Bachhawat, B. K. & Balganes, T. S. (1982a) *Biochem. Biophys. Res. Commun.* **104**, 141–147
- Dorai, D. T., Mohan, S., Srimal, S. & Bachhawat, B. K. (1982b) *FEBS Lett.* **148**, 98–102
- Gardner, K. T., Philips, D. R. & Williams, D. C. (1980) *FEBS Lett.* **113**, 196–200
- Gardner, K. T., Gerrard, J. M., White, J. G. & Williams, D. C. (1981) *Nature (London)* **289**, 688–690
- Jaffe, E. A., Leuang, L. K., Nachman, R. L., Levin, R. I. & Moseher, D. F. (1981) *Nature (London)* **295**, 246–248
- Jorgenson, J. H. & Smith, R. F. (1973) *Appl. Microbiol.* **26**, 43–48
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Levin, J. (1967) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **26**, 1707–1712
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Marachalonis, J. J. & Edelman, G. M. (1968) *J. Mol. Biol.* **32**, 453–465
- Noguchi, H. (1903) *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Hyg. Abt. Orig.* **33**, 352
- Rickles, F. R., Rick, P. D., Armstrong, P. B. & Levin, J. (1979) in *Biomedical Applications of the Horseshoe Crab (Limulidae)* (Cohen, E., ed.), pp. 203–207, Alan R. Liss, New York
- Shishikura, F. & Sekiguchi, K. (1983) *J. Biochem. (Tokyo)* **93**, 1539–1546
- Yeaton, R. W. (1981a) *Dev. Comp. Immunol.* **5**, 391–402
- Yeaton, R. W. (1981b) *Dev. Comp. Immunol.* **5**, 535–545