Isolation and Studies of the Granules of the Amebocytes of *Limulus polyphemus*, the Horseshoe Crab [1,2]

ERIK H. MÜRER,[3,4] JACK LEVIN [5,6,7] AND RANDI HOLME
Marine Biological Laboratory, Woods Hole, Massachusetts 02543; Institute for Thrombosis Research, Rikshospitalet, Oslo, Norway; [5] *The Johns Hopkins University School of Medicine and Hospital, Baltimore, Maryland 21205; SCOR Center for Thrombosis Research, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140 and Electron Microscopic Laboratory, Institute of Pathology, Rikshospitalet, Oslo, Norway*

ABSTRACT Granules were isolated from the cytoplasm of the amebocytes of *Limulus polyphemus*, the horseshoe crab, by disruption of cells obtained from blood which had been drawn into 2 mM propranolol. The granules subsequently were purified by centrifugation through a sucrose gradient that contained heparin. Extracts of the granules were prepared by freezing and thawing the granule preparations in distilled water.

Transmission and scanning electron microscopy of the granules revealed round or ovoid particles. However, only one type of granule appeared to be present. The ultraviolet spectrum of the extract of amebocyte granules demonstrated a peak at 277 nm at pH 7.4, and a shift into two peaks of 281 nm and 290 nm at alkaline pH. Analytical ultracentrifugation revealed a pattern similar to that observed with lysates prepared from intact amebocytes. Polyacrylamide gel electrophoresis, in the presence of urea at pH 4.5, demonstrated patterns similar to those observed with amebocyte lysate. Extracts of the granules were gelled by bacterial endotoxin.

The blood of the horseshoe crab contains only one type of cell, the amebocyte. Previous studies have shown that the blood coagulation mechanism of Limulus is contained entirely within amebocytes. The current studies suggest that the granules, which pack the cytoplasm of these cells, contain all of the factors required for the coagulation of blood, including the clottable protein. The intracellularly localized coagulation system is released from amebocytes when their granules rupture during cell aggregation.

The blood of *Limulus polyphemus*, the horseshoe crab, contains only one type of cell, the amebocyte. Previous studies have demonstrated that the blood coagulation mechanism of *Limulus polyphemus* is contained entirely within the amebocytes (Levin and Bang, '68; Solum, '70). Therefore, blood coagulation in *Limulus polyphemus* is dependent upon disruption of the amebocytes, with subsequent release of the various components of the coagulation mechanism into the surrounding medium (Levin and Bang, '64a, '68; Solum, '70). Coagulation subsequently occurs. Minute concentrations of bacterial endotoxins are capable of activating the coagulation system (Levin and Bang, '64a, '68; Levin et al., '70; Solum, '70). Recent studies have

Received Jan. 20, '75. Accepted Apr. 3, '75.

[1] Supported in part by Research Grants HL 01601 and HL 14217 from the National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014.

[2] Presented in part at the Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1973 (Mürer, E., J. Levin, R. Holme and K. Grøttum 1973 Isolation and examination of granules from Limulus amebocytes. Fed. Proc., 32: 317, abstract).

[3] Dr. Mürer was a recipient of a travel grant under the Senior Fulbright-Hays Act in 1971, and a Research Fellow of the University of Oslo during the early part of this study.

[4] Present address: Erik H. Mürer. SCOR Center for Thrombosis Research, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140.

[6] Dr. Levin is the recipient of a Research Career Development Award (KO 4 HL 29906) from the National Heart and Lung Institute, National Institutes of Health, and was a John and Mary R. Markle Scholar in Academic Medicine during the performance of some of these studies.

[7] Requests for reprints to Dr. Jack Levin, Hematology Division, The Johns Hopkins Hospital, Baltimore, Maryland 21205.

indicated that the clottable protein contained within Limulus amebocytes has a molecular weight of less than 30,000 and that gelation of this coagulable protein occurs after the activation by endotoxin of an enzymatic mechanism (Young et al., '72; Solum, '73).

The cytoplasm of amebocytes is packed with granules (Levin and Bang, '64b, '68; Dumont et al., '66). During coagulation, these granules disrupt, and it has appeared likely that they contain at least some of the components of the blood coagulation mechanism (Levin and Bang, '64a,b; '68; Dumont et al., '66). These studies were performed in order to isolate the granules of Limulus amebocytes, determine some of their characteristics, and establish whether they contain the components of the blood coagulation system in this animal.

MATERIALS AND METHODS

Previous studies have successfully utilized N-ethyl maleimide to prevent the aggregation of Limulus amebocytes (Levin and Bang, '68; Solum, '70). Although this technique proved successful for the preparation of an extract of the intracellular components responsible for blood coagulation, preliminary experiments indicated that intact granules could not be prepared from NEM-treated amebocytes. Caffeine has been shown previously to inhibit aggregation of blood platelets, as well as clot retraction (Ardlie et al., '67). The latter effect is counteracted by adrenaline (Mürer, '71). Although caffeine (Sigma Chemical Co., St. Louis, Mo.), at concentrations of 10–30 mM, also prevented clumping and disintegration of amebocytes, subsequent disruption of caffeine-treated cells resulted in activation of the coagulation system with resultant clotting. The protective effect of caffeine suggested to us that adrenergic activation might be involved in the mechanism of cell disruption. α-adrenergic blockers have been shown previously to inhibit platelet aggregation induced by low concentrations of adrenaline, while significantly higher concentrations of β-blockers were needed, indicating that this latter type of inhibition might be non-specific (Bygdeman, '68). The α-adrenergic blocker, dibenzylene, (Smith, Kline and French Laboratories, Philadelphia, Pa.) did not affect the aggregation or disruption of amebocytes. However, propranolol (Sigma), a β-blocker in most cells, seemed to have a double effect in preventing aggregation and allowing disruption of the cell membrane without concomitant disruption of the cytoplasmic granules. Therefore, the following technique was used to prepare granules of Limulus amebocytes:

One hundred milliliters of Limulus blood was drawn directly into 100 ml of 3% sodium chloride, which contained 2 mM propranolol. The solution had been pre-chilled in wet ice and, after mixing, the blood cells were allowed to settle at 0°C.

The supernatant was discarded by decanting, and the sedimented cells were placed in 10 ml of 0.75 M sucrose in 8 mM Tris-HCl (pH 7.4), which contained 1 mM propranolol. The cells then were resuspended in the sucrose solution by gentle agitation. The suspension was centrifuged at room temperature for five minutes at approximately 250 *g*. The supernatant, which contained granules from ruptured amebocytes, was removed. The yield of granules was increased by resuspension of the precipitated cells in an additional 10 ml of an identical solution of sucrose and propranolol, and the same cycle was repeated two to three times until the resultant supernatants no longer contained significant numbers of granules, as determined by phase microscopy. Electron microscopic studies of granules were performed, utilizing supernatants at this stage in the procedure.

The supernatants then were pooled and 50 units/ml of heparin were added. The suspension of granules subsequently was diluted with an equal volume of 3% sodium chloride, and layered on top of 10 ml of 0.75 M sucrose in water, which also contained heparin (50 units/ml). The tube was centrifuged for one hour at 500 *g* at 4°C. The entire gradient then was removed with a Pasteur pipette and the sedimented pellet of granules was resuspended in 0.5–1 ml of sterile, pyrogen-free distilled water.

The granules then were frozen and thawed, and vigorously agitated on a Vortex mixer for five to ten seconds. Another cycle of freezing and thawing was performed. The studies to be described were performed with supernatants obtained following freezing and thawing of amebocyte granules in distilled water, and removal of

particulate material by centrifugation at 1500 *g* at room temperature.

The UV spectrum of the distilled water extract from granules was determined after dilution with 7 mM Tris-HCl (pH 7.4), with a Beckman DK A_3 spectrophotometer. Spectra of alkaline solutions were obtained after addition of 0.2 M NaOH (one-tenth volume), and again after additional 2 M NaOH (three-hundredths volume).

Ultracentrifugation was carried out in an analytical ultracentrifuge (Beckman Instruments, Fullerton, Cal.), equipped with a Schlieren optical system, at 59,780 rpm, and 21 °C.

Transmission electron microscopy was performed on granules which had been fixed initially in 4% glutaraldehyde in 0.13 M Sørensen's phosphate buffer, pH 7.3, for two hours (1 ml granule suspension to 9 ml glutaraldehyde solution). The granules then were sedimented by centrifugation at 3000 rpm for 30 minutes. The sedimented granules were postfixed in 1% OsO_4 in Tyrode solution for two hours, and dehydrated in ethanol. The entire procedure was carried out at room temperature. The specimen was embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop I electron microscope.

Scanning electron microscopy was performed on granules which had been fixed initially in a freshly mixed solution of 5% glutaraldehyde, 2% osmium tetroxide, and 0.6% sucrose in 0.1 M cacodylate buffer, pH 7.4. Fixation time was ten minutes. The granule pellets were washed in three changes of distilled water and dehydrated in acetone. The acetone was added dropwise until 50% was reached, and then the volume redoubled with acetone to produce a concentration of 75% acetone. Then, this was decanted and replaced with 100% acetone. Following three changes of 100% acetone, the acetone was replaced gradually by increasing concentrations of Freon 113 (trichlorofluoroethane). Finally, 100% Freon was achieved, and replaced twice. The specimens were air dried on glass plates, placed in a dessiccator, and then coated with a thin layer of gold in a vacuum evaporator. The specimens were examined in a Jeolco scanning electron microscope (SM-U3), in the laboratory of Dr. Virginia Peters, Woods Hole Oceanographic Institute.

Polyacrylamide gel electrophoresis was performed, using 7 3/4% gels, in the presence of urea, at pH 4.5, according to the method of Fogel and Sypherd ('68). Extracts of granules were compared with extracts prepared from intact amebocytes (amebocyte lysate), as previously described by Levin and Bang ('68).

E. coli endotoxin (lyophilized lipopolysaccharide of Escherichia coli) was obtained from Difco Laboratories, Inc., Detroit, Michigan. The endotoxin was diluted with pyrogen-free, 0.9% sodium chloride.

The functional characteristics of granule extracts were studied by determining the effects of bacterial endotoxin on these extracts and comparing the reaction with that observed between endotoxin and amebocyte lysate (Levin and Bang, '68; Levin et al., '70). The effect of previously isolated components obtained from amebocyte lysate on the reaction between granule extracts and endotoxin was determined (Young et al., '72).

RESULTS

Transmission electron microscopy of granule preparations, prior to their disruption with distilled water showed ovoid-shaped granules with different electron densities (figs. 1, 2, 3). In granules with high electron densities, the membranes seemed to be intact and the granule material was homogeneous. Membranes in the granules with lower electron densities were partly disrupted and the material in these granules had a particulate or tubular appearance (figs. 2, 3).

Scanning electron microscopy revealed round or ovoid-shaped granules, some of which demonstrated small, irregular protrusions on their surfaces (fig. 4A). Following disruption with distilled water, granule forms often remained apparent, although they were ragged, covered with shaggy-appearing material, and in some instances probably collapsed (fig. 4B).

The ultraviolet spectrum of extracts of amebocyte granules at neutral pH demonstrated a peak at approximately 277 nm (fig. 5). In alkaline solution, the spectrum showed a shift and a slight split into two peaks, at 281 nm and 290 nm, which are characteristic for compounds which contain tryptophane and tyrosine. At both neutral and alkaline pH, the spectrum demon-

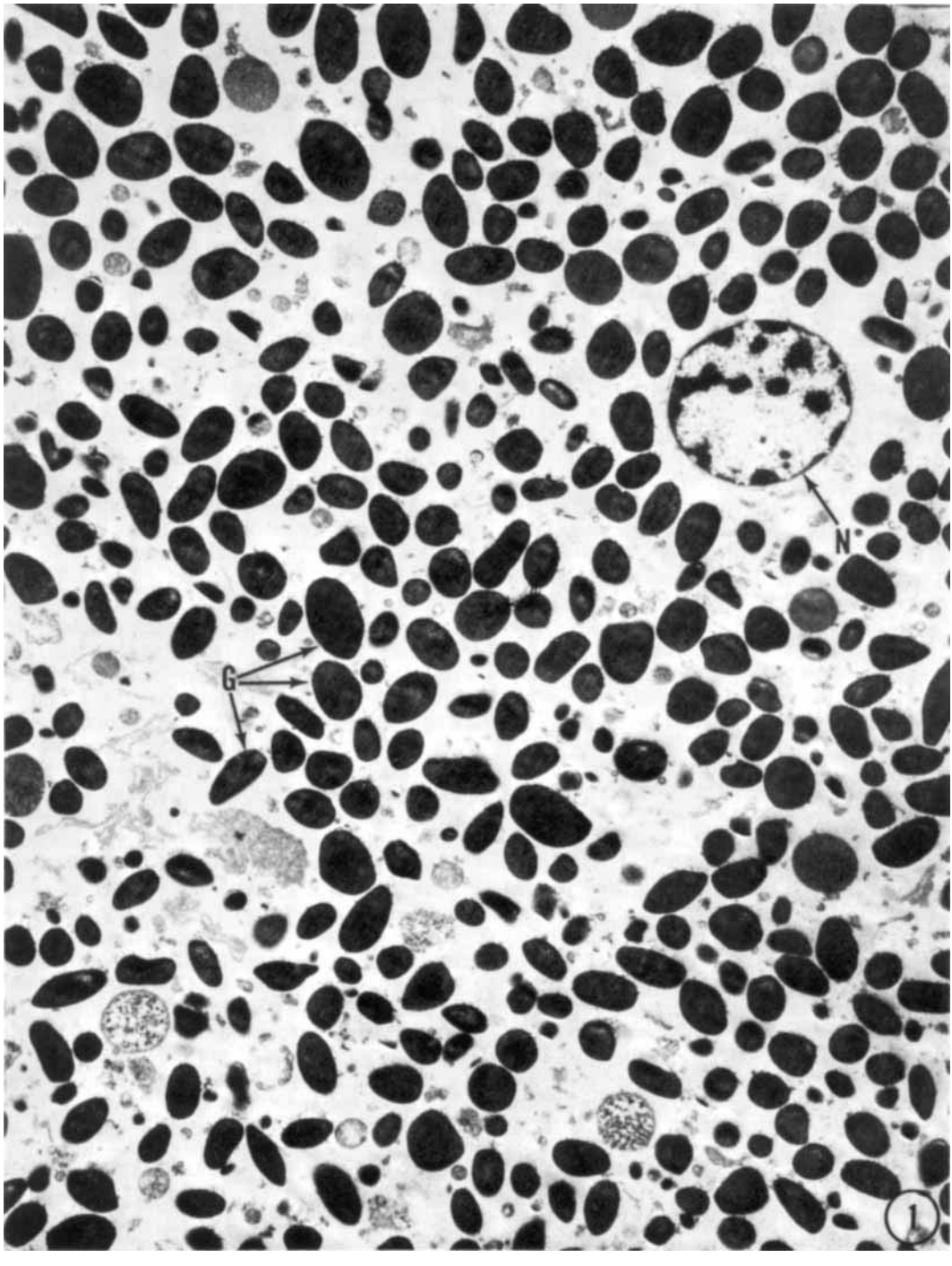
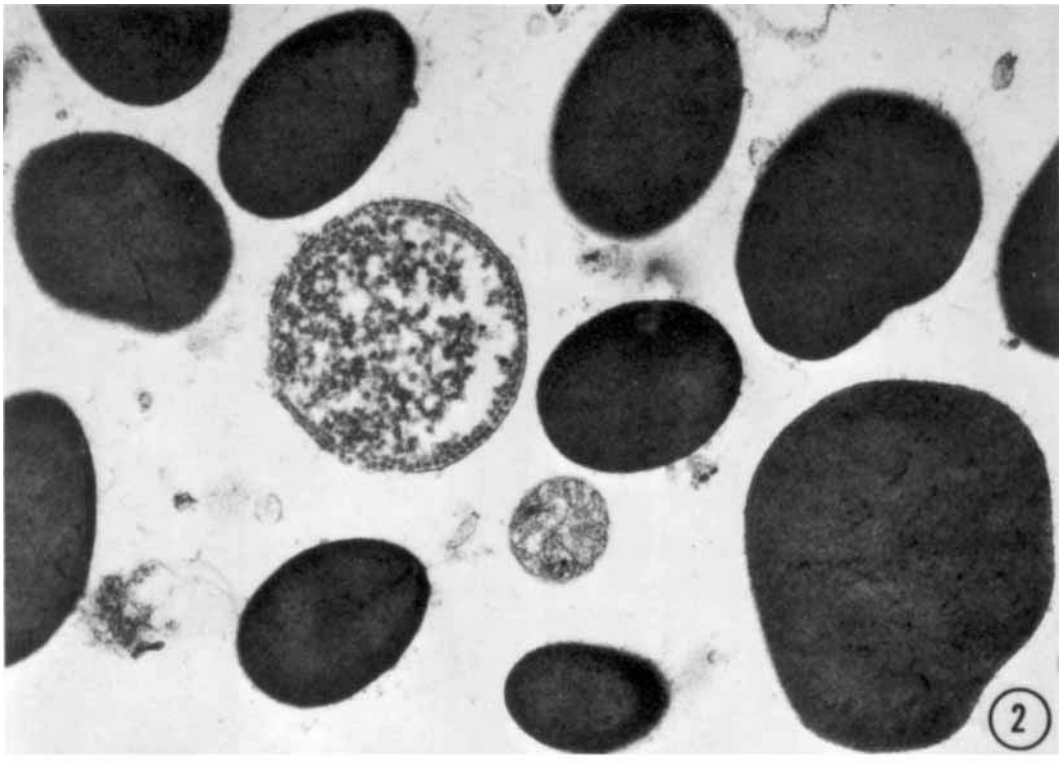


Fig. 1 Transmission electron micrograph of cytoplasmic granules (G) in the granule-rich supernatant obtained from propranolol-treated amebocytes (MATERIALS AND METHODS). A nucleus (N) also was observed. Final magnification is × 6,000.



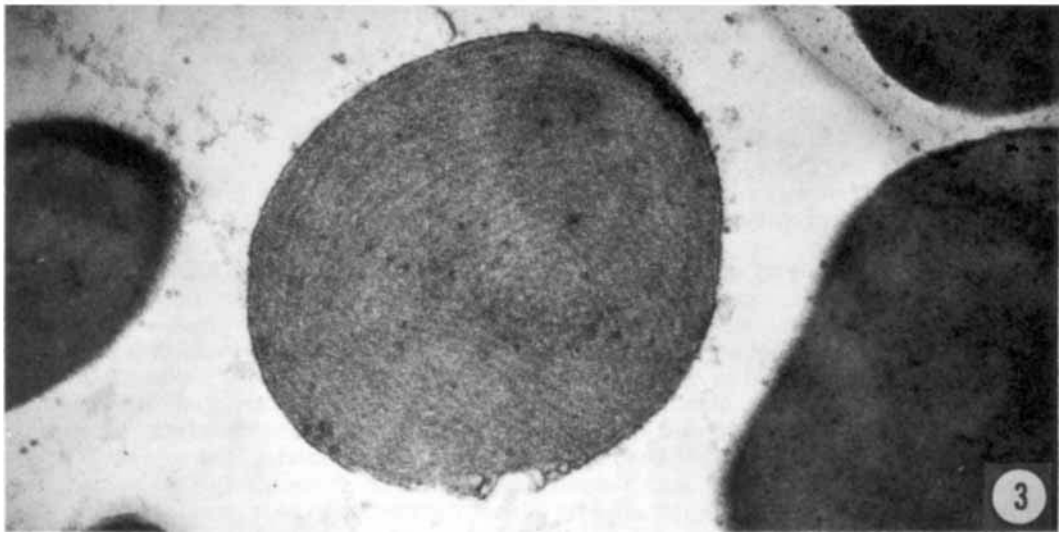


Fig. 2 Granules from the same preparation as in figure 1, at a higher magnification. A swollen granule with disrupted membranes is present. Final magnification is × 24,000.

Fig. 3 Granule from the same preparation as above. Note the tubular structure. Final magnification is × 40,000.

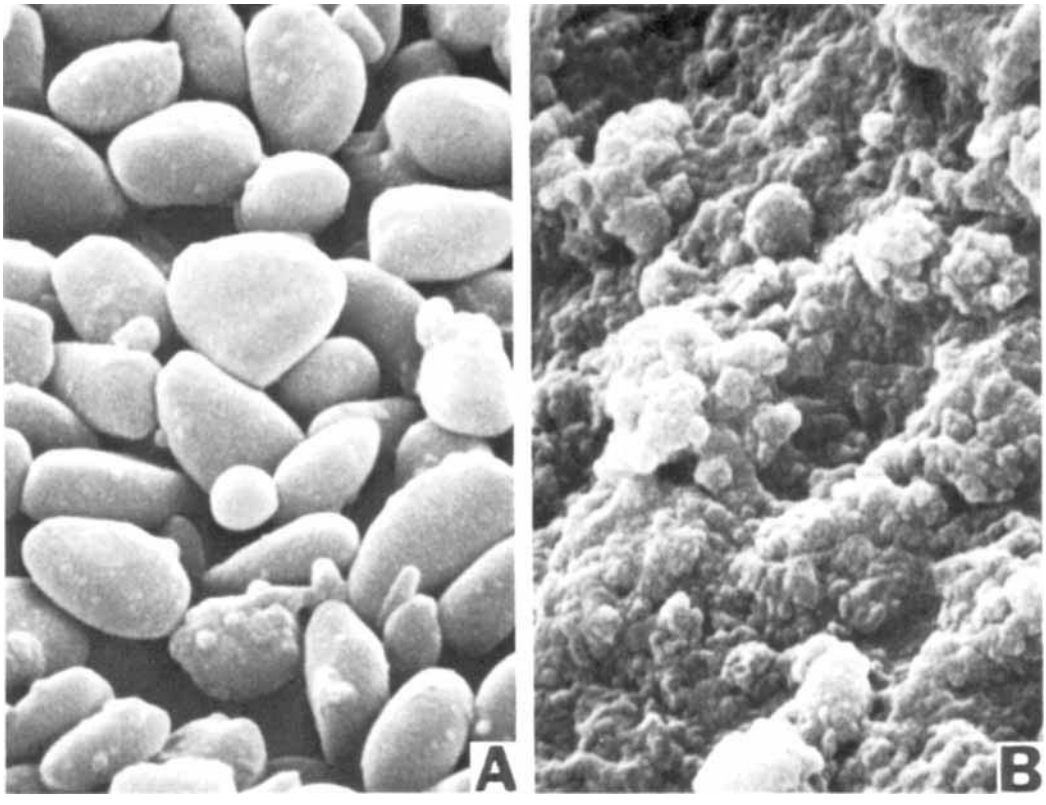


Fig. 4 Scanning electron micrographs of cytoplasmic granules obtained from Limulus amebocytes. A (left): Intact Granules. B (right): Following disruption in distilled water. Final magnification is × 10,000.

strated a peak at 319 nm and an irregular shoulder at 305 nm. These conformed to the spectrum of propranolol and were probably caused by a residue of this compound in the preparation.

Analytical ultracentrifugation of the granule extract demonstrated a pattern similar to that observed with lysates prepared from intact amebocytes (fig. 6). Three peaks were observed in both types of preparations, although the concentration of material was lower in the granule extracts. Comparison with previously published studies suggested that the third and largest peak contained the clottable protein (Levin and Bang, '68).

Polyacrylamide gel electrophoresis revealed a pattern similar to that observed with extracts of whole lysate although some minor components were not evident in the granule extracts (fig. 7). The major, fast-moving band has been shown to be the clottable protein (Levin, unpublished observations).

The extracts of the granules were clear and colorless. Protein concentrations in different preparations ranged from 1 to 4 mg/ml. Following addition of bacterial endotoxin, a solid gel formed within 15–60 minutes. Further evidence that granule extracts contained clottable protein was provided by a technically unsuccessful experiment, in which the granules disrupted during centrifugation of the sucrose gradient. Strands of protein were formed. Light microscopic examination of these strands indicated that many granules were trapped within the coagulum. The only significant source of protein present in this preparation was the suspension of granules which had been initially layered on top of the gradient.

DISCUSSION

Granules were obtained from the cyto-

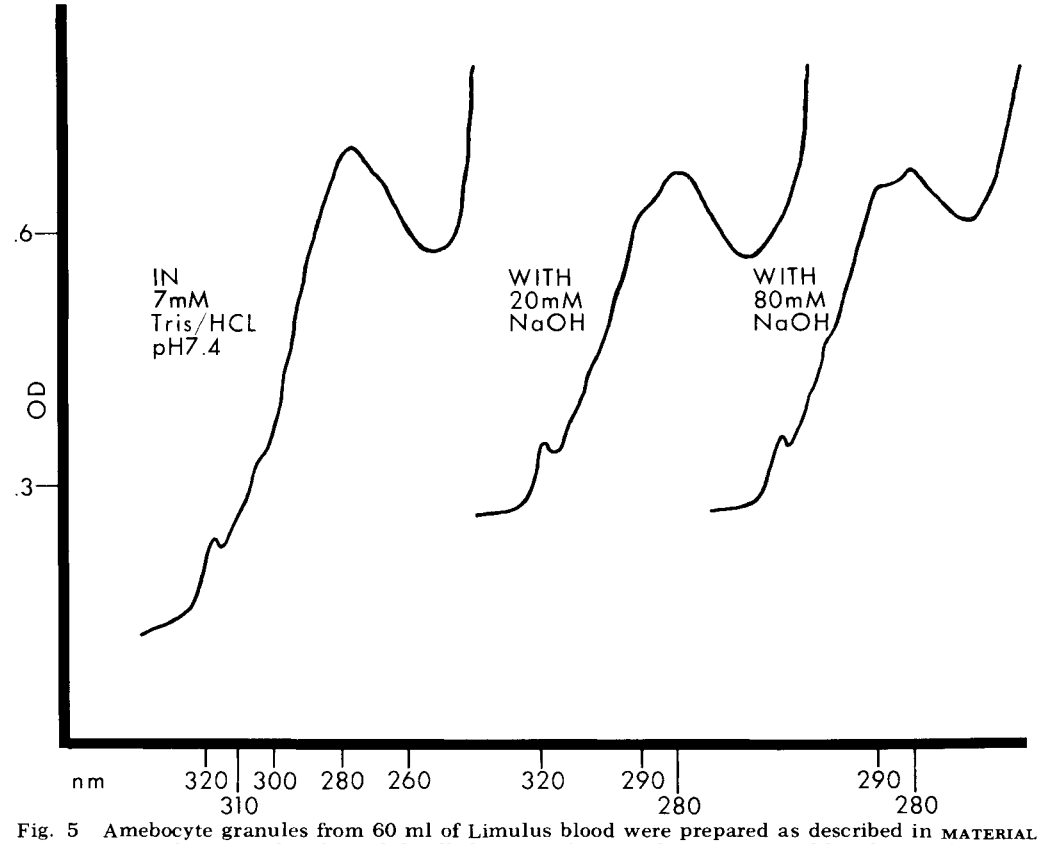


Fig. 5 Amebocyte granules from 60 ml of Limulus blood were prepared as described in MATERIALS AND METHODS, and extracted with 1 ml distilled water. The granule extract was diluted to 4 ml in 7 mM Tris-HCl (pH 7.4) and the spectrum measured in the presence of increasing concentrations of NaOH, as described in MATERIALS AND METHODS.

plasm of Limulus amebocytes by disruption of the amebocytes in a solution of propranolol and sucrose, and subsequent isolation of granules by centrifugation through a layer of sucrose which contained heparin. Although electron microscopic examination of granules prepared in this manner revealed some heterogeneity in size and shape, it appeared that there was only one type of granule in the cytoplasm of Limulus amebocytes. Dumont et al. ('66) reported similar observations.

Studies were performed using supernatants obtained following the freezing and thawing of amebocyte granules in distilled water. The ultraviolet absorption spectrum of extracts of amebocyte granules did not indicate significant contamination with nuclear components. The absence of an absorption peak at 271 nm in the granule extracts, in contrast to the presence of this peak in extracts of whole cells, indicated the absence of nucleotides of cytoplasmic or nuclear origin. Lactic dehydrogenase, a marker for extra-granular cytoplasmic material, was not detected in significant concentrations in extracts of granules. Therefore, contamination of the granule extracts by cytoplasmic proteins did not appear to be likely.

Analytical ultracentrifugation, polyacrylamide gel electrophoresis, and evaluation of the biological activity of extracts of granules indicated that the granules contained the clottable protein present in Limulus amebocytes, and probably the entire intracellularly located coagulation mechanism of the horseshoe crab. The reaction between bacterial endotoxin and granular extracts produced a solid gel, as is the case when bacterial endotoxin is reacted with extracts of Limulus amebocytes (Levin and Bang,

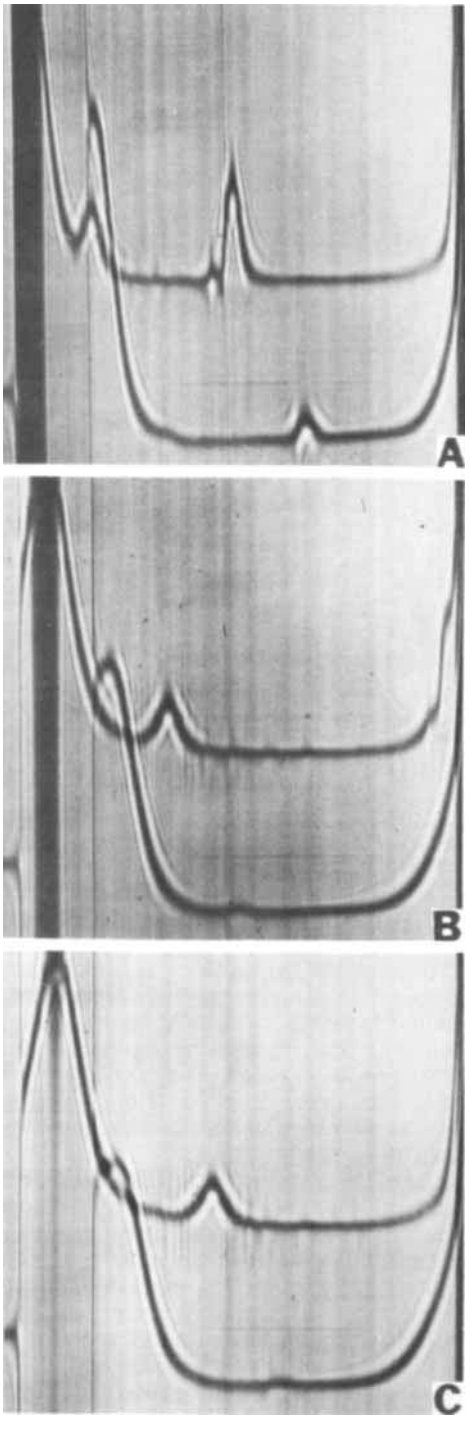


Figure 6

'64a, '68; Levin et al., '70). Dumont et al. ('66) also have proposed that amebocyte granules contain coagulation factors. However, in some experiments, the rate of the reaction between the extract of granules and bacterial endotoxin was increased by the addition of fraction I of amebocyte lysate, which previously had been shown to contain an enzyme or series of enzymes which increase the rate of the reaction between the clottable protein of amebocytes and bacterial endotoxin (Young et al., '72). This suggests that some materials leaked from the granules during their preparation. The tendency of the granules to leak during their isolation also was indicated by the lack of ability to obtain a functioning coagulation system (as measured by its ability to gel following exposure to endotoxin) from some preparations, despite the presence of apparently intact and morphologically normal granules. Nevertheless, it is possible that some components of the coagulation mechanism of Limulus are contained in the extra-granular cytoplasm of amebocytes.

There are important dissimilarities between amebocytes and mammalian blood cells. The Limulus amebocyte, by virtue of its important role in blood coagulation, serves as a significant factor both in hemostasis and control of bacterial infection. However, it appears that the granules of these cells primarily contain the blood coagulation mechanism, rather than the hydrolytic enzymes associated with the granules of blood cells primarily concerned with phagocytosis and the destruction of bacteria (Baggiolini, '72). Preliminary studies were carried out to evaluate the possible presence (in amebocyte granules) of enzymes known to be contained in lysosome-rich granules of the granulocytes of other

Fig. 6 Ultracentrifugation patterns of a lysate of Limulus amebocytes compared with an extract of cytoplasmic granules. The upper cell contained amebocyte lysate and the lower cell contained an extract obtained from granules. Time after the full speed of 59,780 rpm had been reached: A (11 min); B (21 min); C (27 min).

Three peaks are evident in each preparation, and the patterns are similar. The direction of sedimentation is rightward. The fastest moving material (first peak) has disappeared from both preparations in panels B and C. The material which moved slowest and was in the highest concentration represents the clottable protein.

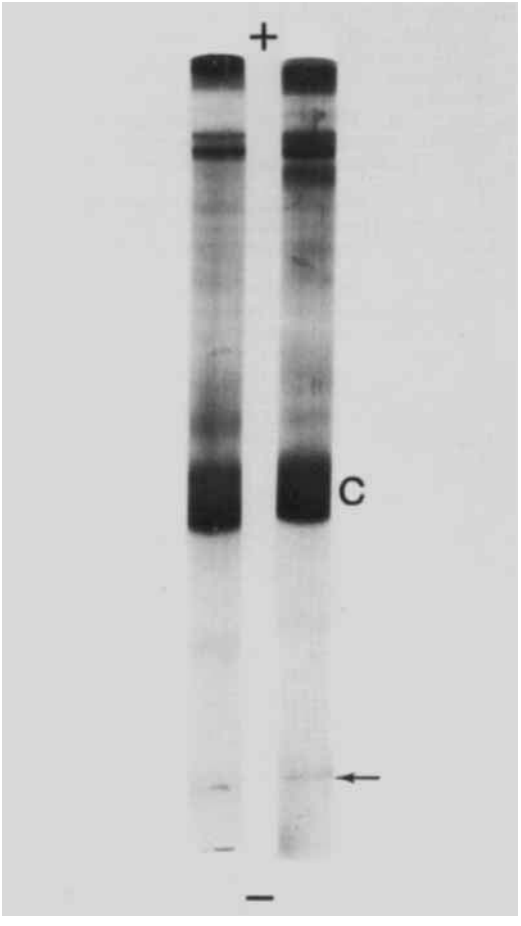


Fig. 7 Electrophoretic patterns of an extract of the cytoplasmic granules (left) and of amebocyte lysate (right) in 7 3/4% polyacrylamide gels. Gels contained urea at pH 4.5 and were stained with 0.25% Amido Black. The migration of proteins was from positive to negative. C (clottable protein); Arrow (position of tracking dye).

animals (Weissmann et al., '72). The results of these experiments indicate that it is highly unlikely that the granules which contain the coagulation mechanism of Limulus also contain lysosomal beta-glucuronidase.[8]

Human blood platelets have been shown to contain fibrinogen, a significant proportion of which is localized in the storage granules of these cells (Nachman and Marcus, '68; Day and Solum, '73). However, fibrinogen is only one of many compounds released from platelets (Holmsen et al., '69), in contrast to the apparent homogeneity of the granules of amebocytes. Belamarich et al. ('73) and Dumont et al. ('66) have suggested that amebocytes, in vivo, discharge their contents at the cell membrane, with the granule membranes remaining intracellularly.

The Limulus amebocyte displays distinct differences, both in structure and function, from the mammalian blood platelet. The degree to which our data reflect general differences between the blood cells of vertebrates and invertebrates cannot be determined from currently available information.

ACKNOWLEDGMENTS

Part of this study could not have been performed in Norway without the cooperation of Dr. Finn Walvig at the University of Oslo Marine Biological Station, Drøbak, Norway. Dr. Kjell A. Grøttum, presently at the University of Tromsø, Norway, gave us valuable help and advice in the early stages of the study.

Mr. Robinson Hyde assisted in the performance and interpretation of the ultracentrifugation studies. Dr. Virginia Peters aided in the preparation and interpretation of the scanning electron micrographs.

LITERATURE CITED

Ardlie, N. G., G. Glew, B. G. Schultz and C. J. Schwartz 1967 Inhibition and reversal of platelet aggregation by methyl xanthines. Thrombos. Diathes. Haemorrh., *18*: 670–673.

Baggiolini, M. 1972 The enzymes of the granules of polymorphonuclear leukocytes and their functions. Enzyme, *13*: 132–160.

Belamarich, F. A., R. A. Stiller and D. Shepro 1973 The release reaction: is it a common mechanism in all types of hemostatic cells? Series haematologica, *6*: 418–428.

Bygdeman, S. 1968 Effect of α- and β-receptor blocking agents on platelet aggregation and uptake of noradrenaline. Acta Physiol. Scand., *74*: 28–29A.

Day, H. J., and N. O. Solum 1973 Fibrinogen associated with sub-cellular platelet particles. Scand. J. Haemat., *10*: 136–143.

Dumont, J. N., E. Anderson and G. Winner 1966 Some cytologic characteristics of the hemocytes of Limulus during clotting. J. Morph., *119*: 181–217.

Fogel, S., and P. S. Sypherd 1968 Extraction and isolation of individual ribosomal proteins from Escherichia coli. J. Bact., *96*: 358–364.

Holmsen, H., H. J. Day and H. Stormorken 1969

[8] These studies were carried out by Miss Linda Crouse and Mr. Stuart Lind in the laboratory of Dr. Gerald Weissmann at the Marine Biological Laboratory.

The blood platelet release reaction. Scand. J. Haemat., Supplement, 8: 1–26.

Levin, J., and F. B. Bang 1964a The role of endotoxin in the extra-cellular coagulation of Limulus Blood. Bull. Johns Hopkins Hosp., *115*: 265–274.

——— 1964b A description of cellular coagulation in the Limulus. Bull. Johns Hopkins Hosp., *115*: 337–345.

——— 1968 Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thrombos. Diathes. Haemorrh., *19*: 186–197.

Levin, J., P. A. Tomasulo, R. S. Oser 1970 Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med., 75: 903–911.

Mürer, E. H. 1971 Compounds known to affect the cyclic adenosine monophosphate level in blood platelets: Effect on thrombin-induced clot retraction and platelet release. Biochim. Biophys. Acta., 237: 310–315.

Nachman, R. L., and A. J. Marcus 1968 Immunological studies of proteins associated with the subcellular fractions of thrombasthenic and afibrinogenaemic platelets. Brit. J. Haemat., *15*: 181–189.

Solum, N. O. 1970 Some characteristics of the clottable protein of Limulus polyphemus blood cells. Thrombos. Diathes. Haemorrh., 23: 170–181.

——— 1973 The coagulogen of Limulus polyphemus hemocytes. A comparison of the clotted and non-clotted forms of the molecule. Thrombosis Res., 2: 55–70.

Weissmann, G., R. B. Zurier and S. Hoffstein 1972 Leukocytic proteases and the immunologic release of lysosomal enzymes. Amer. J. Path., 68: 539–562.

Young, N. S., J. Levin and R. A. Prendergast 1972 An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. J. Clin. Invest., *51*: 1790–1797.