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# CRUSTACEAN HAEMOCYTE REACTIVITY TO CYTOCHALASIN B

by

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SUMMARY

The characteristic features of haemocytes reacting to cytochalasin B are as follows :

1) threadlike pseudopodial elongations develop at one or both ends of the spindle shaped cells while the rest of the cytoplasm shows a contracted state;
2) rows of vacuoles are lined under the surface membrane; many granules are moving forward and backward within the pseudopods, others dissolve in the vacuoles or remain clustered around the nucleus;
3) the reactions are dose-dependent, require the presence of calcium ions and are inhibited by low temperatures;
4) colchicine and nocodazole inhibit the polarization of pseudopod emission as well as the translation of granules;
5) disruption of the microfilament network is discussed to explain the observed reactions.

## INTRODUCTION

Crustacean haemocytes have been divided into three basic types according to the load of granules in the cytoplasm. Hyalin cells have none or few small granules while granular cells are packed with large granules. Semi-granular cells represent an intermediate stage of differenciation between the two other cell types.

The three haemocyte types are committed to a wide range of physiological activities such as phagocytosis, capsule and nodule formation, wound repair and blood clotting. Under these circumstances, the cytoplasm undergoes several morphological changes, the basic mechanisms of which are still poorly understood (Bauchau, 1981).

Therefore it is interesting to submit crustacean blood cells to biochemical agents known to interfere in a fairly specific way with normal cytoplasmic capabilities of invertebrate as well as vertebrate cells (Jones and Partridge, 1974).

In the present paper an attempt was made to examine how cytochalasin B, colchicine, nocodazole and $Ca^{++}$ affect the actual machinery of haemocyte reactivity.

(*) The present communication is dedicated to Prof. Dr. H. J. Koch who has guided our first steps in the experimental study of crustacean biology.

## MATERIAL AND METHODS

Three species of Crustacea were used. *Carcinus maenas* LINNE was collected on the belgian seashore; *Eriocheir sinensis* H. M. EDWARDS was captured in the Schelde. Both were kept in the laboratory in running seawater at room temperature. *Homarus americanus* H. M. EDWARDS, flown from Canada, was kept in a cold room at 3-5° C.

Haemolymph samples were withdrawn with a syringe from blood sinuses at the base of a pereiopod or directly from the pericardial cavity. In some instances, the carpus of a walking leg was cut off and drops of blood were immediately collected in a convenient solution.

The following solutions were used : a stock solution of cytochalasin B (cyto. B, Aldrich) at 1 mg/ml DMSO (dimethylsulfoxide) diluted to the desired concentration before use; colchicine (Sigma) $2.10^{-2}$M in phosphate buffer 0.1 M at pH 7; nocodazole (Aldrich) 10 µg/ml; EDTA 5 %; phosphate buffer solutions 0.1 M at different pH.

Haemocyte preparation for examination with E.M. Philips 300 was carried out as described in BAUCHAU and DE BROUWER (1972).

Haemocyte preparation for S.E.M. was made according to ANDERSON (1951) with slight modifications. Blood samples were collected in pollyallomer tubes containing glutaraldehyde 2.5 %, NaCl 3 % and phosphate buffer 0.1 M, pH 7; haemocytes were recuperated by filtration on Uni-Pore polycarbonate membrane Bio-Rad (3 µm pore size); fixation was made at 0° C for 30 min.; dehydration was carried out by successive ethanol baths (50° — 75° — 90° — 100° each 15 min.); the haemocytes were washed with propylene oxide; the critical point was prepared by carbon dioxide; coating was made with gold-palladium and examination with JEOL JSM 35.

## RESULTS

### Haemocyte reaction to cyto. B

Haemocytes will readily spread on to a glass surface, project pseudopods in all directions and make contact with adjacent cells. Simultaneously cytoplasmic vesicles are emptied into the plasma which becomes gelified (BAUCHAU and DE BROUWER, 1974). Clusters of cells will form in the same way when a blood sample is diluted with an equal volume of NaCl 3 % buffered at pH 7.

However, addition of 10 to 15 µg/ml cyto. B almost immediately elicits striking morphological changes which become conspicuous within 5-10 min.

First of all the size of the treadlike pseudopodial elongations is greatly enhanced; some of them, which may subsequently subdivide, are 5 to 6 times longer than the mother-cell (Pl. IA). The reaction is shared by hyalin, semi-granular and granular cells and produces an intricated lattice of branching cytoplasmic extensions (Pl. IIB). Amoeboid locomotion is replaced by an at random rotation of the cell body indicating a loose attachment to the substrate.

The pseudopods habitually show a clearly definite polarity, protruding at only one side or at two opposite sides of the cell-margin (Pl. IA). They may be moving slowly or sometimes display brief sudden jerks but do not stick to nearby located cells. Seldom they withdraw into the cytoplasm and disappear.

Another difference lies in the mobility of granules within the pseudopods. During a regular clotting process, they may invade the proximal part of a pseudopod

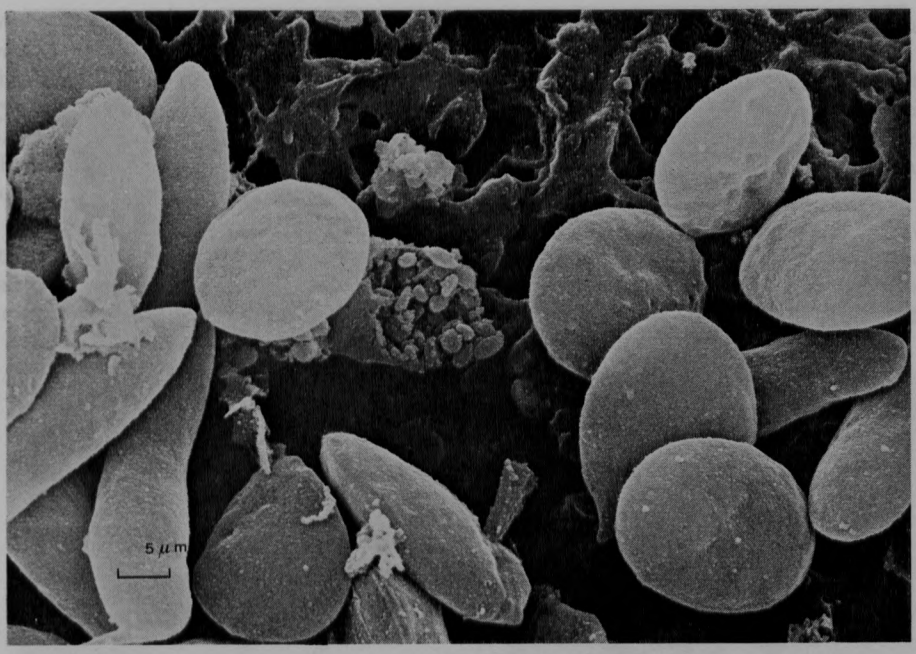


PLATE I A
Phase contrast photograph of haemocytes of *Carcinus maenas* reacting to 10 μg/lm cyto. B. The polarization of long threadlike pseudopods is conspicuous.

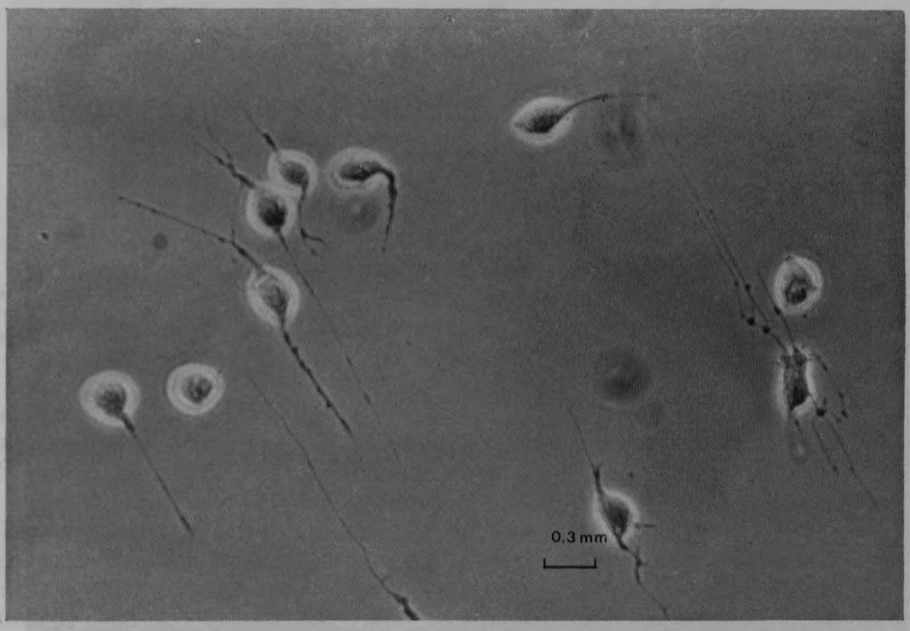


PLATE I B
Phase contrast photograph of a granulocyte of *Carcinus* with bulging granules distributed along a branching pseudopod. Reaction to 10 μg/ml cyto B.

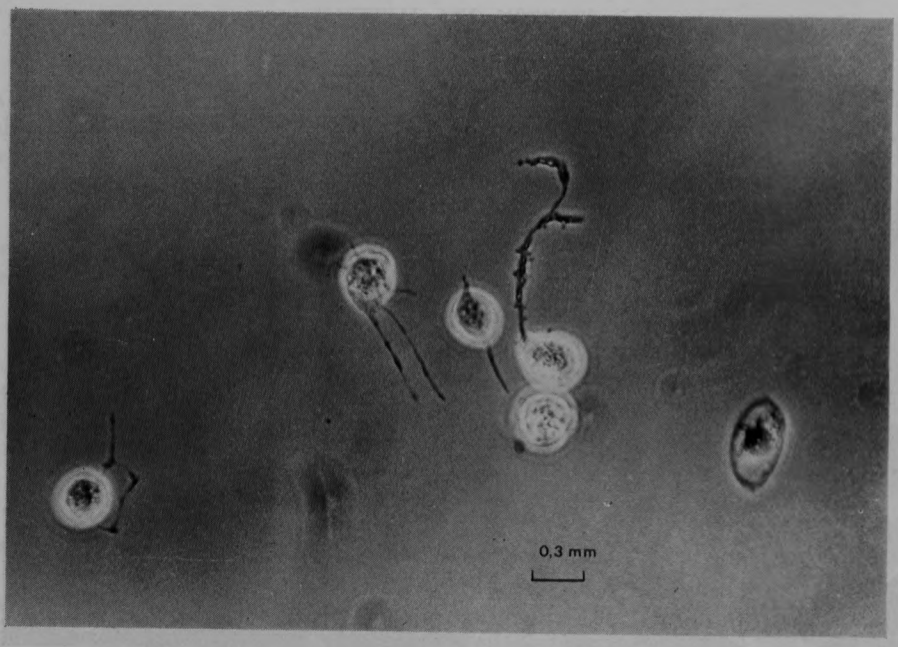


PLATE II A
Scanning photograph of a sample of *Carcinus* haemolymph mixed with an equal volume of EDTA 5 %. Clotting processes are perfectly inhibited and haemocytes retain their regular appearance.

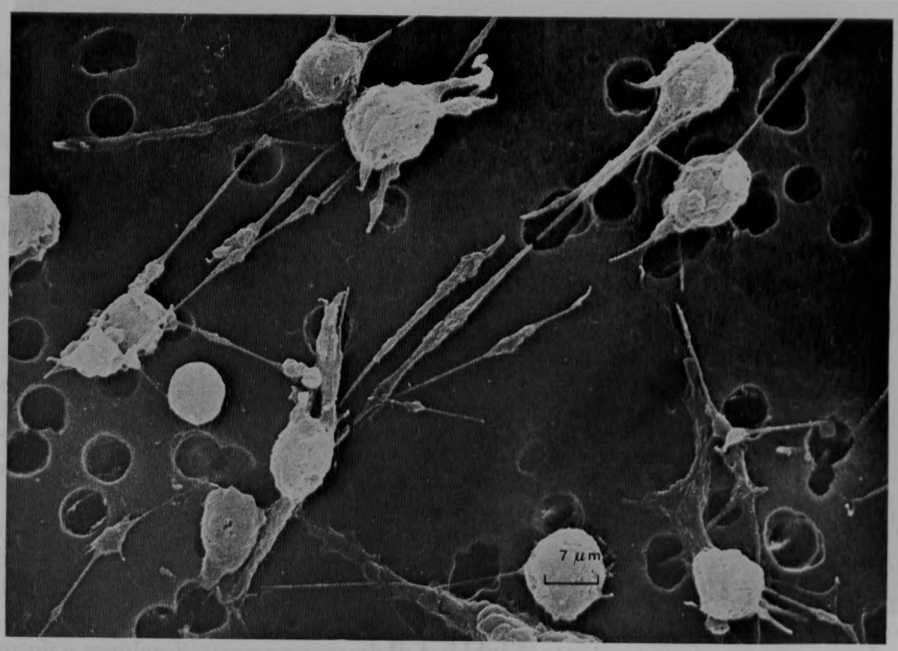


PLATE II B
Scanning photograph of a lattice of branching cytoplasmic extensions of *Carcinus* haemocytes after 10 µg/ml cyto B application.

but will not proceed any further (BAUCHAU and DE BROUWER, 1974). In presence of cyto. B on the contrary, they travel along the main axis and may even reach its tip; they keep moving forward and backward; occasionally they re-enter into the cytoplasm. A release in the medium has never been observed. Up to 10 granules have been counted at the same time in a single pseudopod although others remained clustered around the nucleus. Their bulging mass gives the pseudopod a typically coarse appearance (Pl. IB).

Electron microscopy points to a fourth difference. At the beginning of a reaction to cyto. B, rows of large vesicles are lined under the cell membrane. Some contain one or a few dissolving granules but the majority are filled with a translucent fluid or a light material produced by the dissolution of a granule (Pl. IIIA). When the

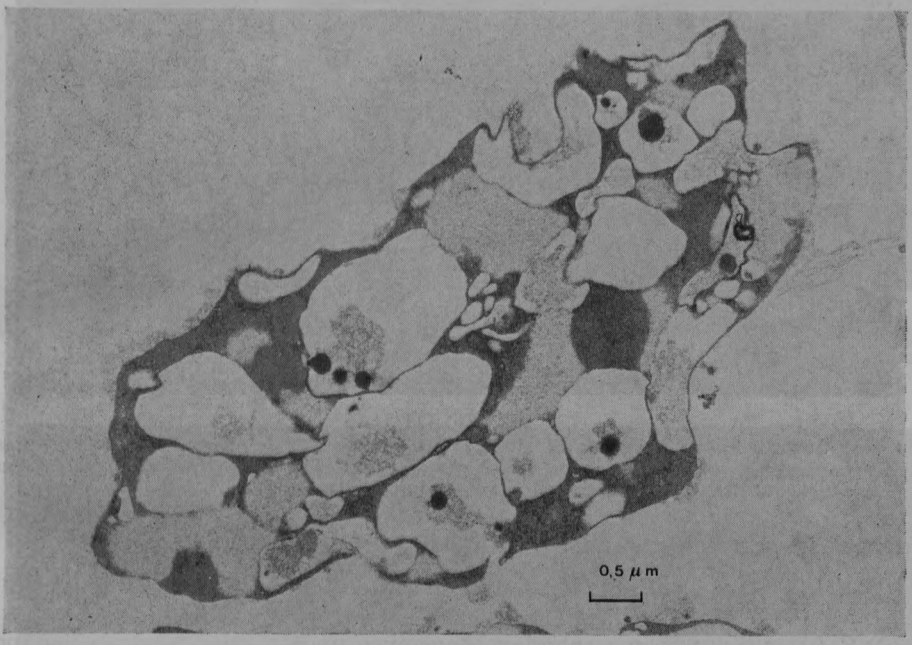


PLATE III A

Parasagittal section of a *Carcinus* granulocyte after 10 µg/ml cyto. B application. The cytoplasm is in a contracted state (dark areas) but is distorted by a great number of large vacuoles. They are filled with a translucent fluid or a light material produced by dissolving granules.

reaction is completed, the cell shows a striking dendriform appearance while the bulk of the cytoplasm is electron-dense, reflecting a generalized retracted state (Pl. IIIB).

At lower cyto. B concentrations (5,1 and 0.1 µg/ml), hyalin and semi-granular cells still display the above described changes while a number of congregated smooth-edged blebs erupt at the cell-margin of granulocytes. Each knob encloses a granule. This mulberry-like shape has been named zeiosis (GODMAN *et al.*, 1975; GODMAN and MIRANDA, 1978).

At even lower concentrations (0.01 µg/ml and 0.001 µg/ml), any typical reaction vanishes but the usual spreading and agglutination of haemocytes still persist.

The threshold concentration for hyalin and semi-granular cells seems to be slightly above 0.01 μg/ml; granulocytes require a higher concentration of about 0.1 μg/ml.

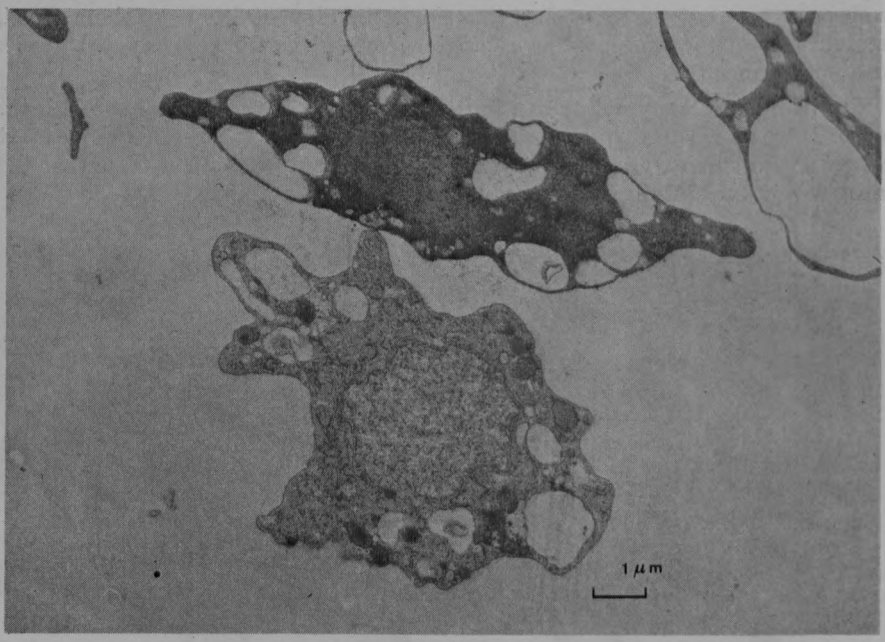


PLATE III B

Section of two semi-granular haemocytes from *Carcinus*. The electron-dense cytoplasm is in a contracted state, with vacuoles and the proximal part of polarized pseudopods (top cell). A less advanced stage of reaction to 10 μg/ml cyto. B (lower cell).

**Interaction between cyto. B, EDTA, temperature and pH**

Clotting of the blood is hampered when a haemolymph sample, diluted with an equal volume of NaCl 3 %, is brought to final pH values ranging from 4.5 to 6. At 0° C, haemocytes keep a spherical shape even upon a glass substrate; at room temperature (20° C), a characteristic spindle shape appearance is common.

Under these conditions, reaction to cyto. B is also inhibited. At pH 6.5, some slight pseudopodial elongations erupt from the cytoplasm but the typical spidery appearance requires a pH of 7 or higher.

Blood clotting is inhibited or at least delayed for several hours by adding a solution of EDTA 5 % to the haemolymph sample (Pl. IIA); the spindle shape also does not materialize, even in the presence of cyto. B. When haemocytes are first subjected to the action of cyto. B and EDTA is subsequently added, haemocytes resume a normal spherical form with occasionally a few short pseudopods.

The reaction to cyto. B appears to be perfectly reversible. It may also be concluded that to be effective cyto. B requires the presence of calcium ions.

**Interaction between cyto. B, colchicine and nocodazole**

Colchicine and nocodazole, two microtubule inhibitors, do not interfere with the usual cytoplasmic spreading, stellate pseudopodial projections and cell agglutina-

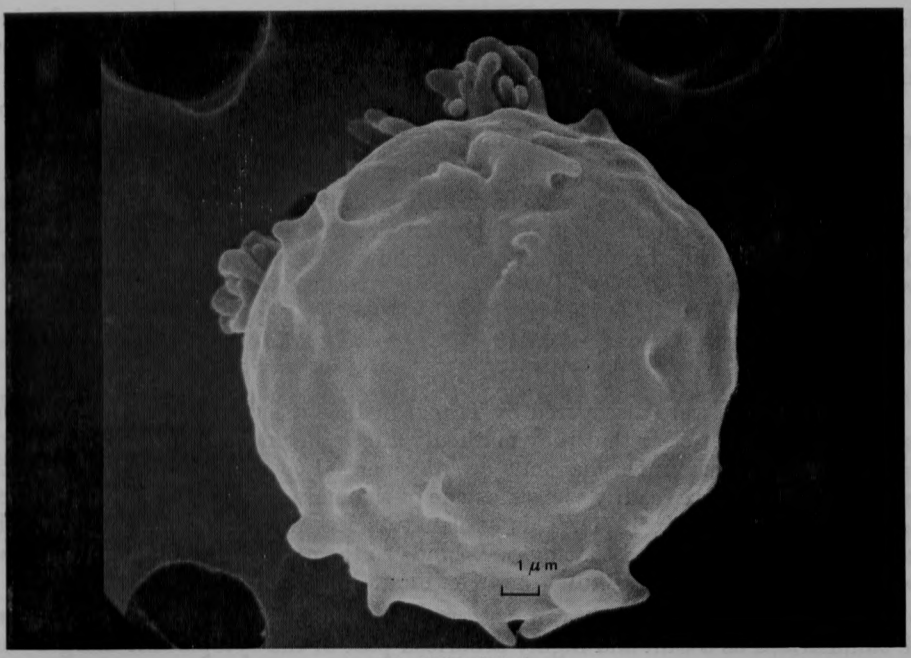


PLATE IV A

Scanning photograph of a *Carcinus* haemocyte after simultaneous addition of 10 μg/ml cyto. B and 10 μg/ml nocodazole. Shorter pseudopods and slighter surface ruffling point to an inhibitory action of nocodazole,

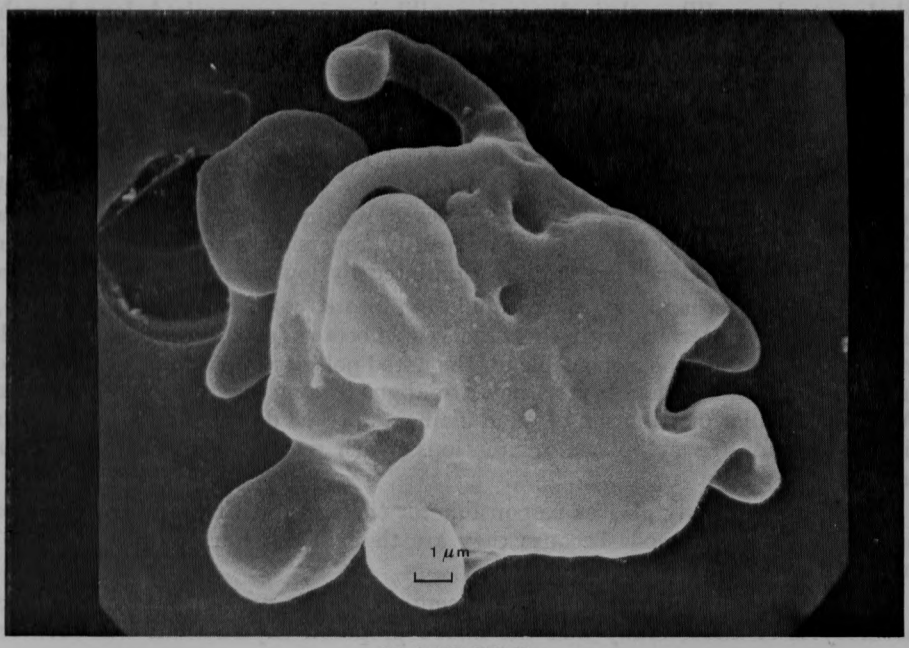


PLATE IV B

Same conditions as for A. Surface distortion and pseudopod elongation are clearly reduced in comparison with Pl. II B.

tion of a blood sample taken out of the haemocoel. However, the absence of spindle shaped haemocytes is conspicuous and motionless granules remain clustered at the center of the cell body (Pl. IV A and B).

Subsequent addition of cyto. B will not induce any polarity in pseudopodial protrusion, nor will it restore a typical mobility of the granules.

When haemocytes are subjected to the fungal metabolite before the introduction of colchicine or nocodazole, the polarized arborization of the cytoplasm persists, but granule translocation is progressively impaired. These data mean that morphological changes initiated by cyto. B are somehow related to the microtubule framework.

## DISCUSSION

Cytochalasins were discovered by Turner in 1964 (Carter, 1972). Experimental probes soon made it clear that they markedly affected contractile processes in a wide variety of cells. Since microfilaments are an essential component of the contraction machinery, they were readily viewed as one of the main targets of the fungal metabolites (Wessels *et al.*, 1971). This explanation has further been confirmed when it was recently established that cyto. B binds to the actin at the filament end where assembly is taking place (Brown and Spudich, 1981).

The characteristic features of crustacean haemocyte response to cyto. B may be summarized as a thin and highly arborized part of the cytoplasmic margin and a thickened centrosphere region. The spectacular morphological transformations may be hypothesized to result from a more or less extensive disruption of the microfilament lattice. Indeed these subcellular organelles not only subserve contractile functions but with microtubules they provide the basic cytoskeletal elements of the whole cytoplasm. When their dynamic equilibrium is compromised, long branching extensions radiate from a contracted cell body. Further the cytoplasmic fluid moiety will be pushed towards the periphery of the cell where indeed a great number of enlarged vacuoles are congregated.

An increase in the concentration of calcium ions in the cytoplasm is the usual trigger for the acto-myosin contractile system. It is of particular interest to notice that any response to cyto. B vanishes when the $Ca^{++}$-chelating agent EDTA is added to the haemolymph. At once haemocytes regain their normal spherical shape. Changes induced by cyto. B appear readily reversible and calcium-dependent. They are also dose-dependent and are inhibited by low temperature (5° C or less).

On the other hand two microtubule inhibitors, colchicine and nocodazole, at least indirectly interfere with the observed effects of cyto. B application. Indeed microtubules seem to be responsible for the noted polarity of cytoplasmic projections. In a spindle shaped haemocyte, they are aligned along the main axis of the cell. When the regular distribution of microfilaments breaks down, they provide a directional force for the cytoplasmic extensions which indeed develop at one or both ends of the spindle.

Microtubules may also be responsible for the sustained mobility of granules in newly formed pseudopods because they are thought to offer guiding channels for translocation of intracellular organelles.

Direct vizualisation of the microfilament network in normal and cyto. B affected haemocytes is under study and the above proposed explanations will further be tested.

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