Olcott, H. S. & Fraenkel-Conrat, H. (1947). *J. biol. Chem.* **171**, 583.
Rimington, C. (1929). *Biochem. J.* **23**, 41.
Ross, D. A. (1961). *Proc. N.Z. Soc. Anim. Prod.* **21**, 153.
Ryder, M. L. (1956). *Nature, Lond.*, **178**, 1409.
Schöberl, A. (1959). *Textil-Praxis*, **7**, 701.
Schöberl, A. (1960). *Melliand Textilber.* **41**, 188.
Schöberl, A. & Rambacher, P. (1940). *Biochem. Z.* **306**, 269.
Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.
Shinohara, K. (1935). *J. biol. Chem.* **109**, 665.
Simmonds, D. H. (1954). *Aust. J. biol. Sci.* **7**, 98.
Thompson, E. O. P. (1955). *Proc. int. Wool Text. Res. Conf. Australia*, C102.
Ward, W. H., Binkley, C. H. & Snell, N. S. (1955). *Text. Res. J.* **25**, 314.
Ward, W. H. & Lundgren, H. P. (1954). *Advanc. Protein Chem.* **9**, 243.
Wibaux, G., Mazingue, G. & Van Overbèke, M. (1960). *Bull. Inst. text. Fr.* **86**, 41.
Zahn, H., Kunitz, F.-W. & Hildebrand, D. (1960). *J. Text. Inst.* **51**, T740.
Zahn, H., Weigmann, H.-D. & Nischwitz, E. (1961). *Kolloidzschr.* **179**, 49.

Biochem. J. (1963) **87**, 567

Morphological Changes Associated with the Extrusion of Protein Induced in the Polymorphonuclear Leucocyte by Staphylococcal Leucocidin

BY A. M. WOODIN, J. E. FRENCH AND V. T. MARCHESI*
Sir William Dunn School of Pathology, University of Oxford

(*Received* 17 *December* 1962)

A survey of biochemical changes that occur when polymorphonuclear leucocytes are treated with leucocidin showed that although the normal impermeability of the cell membrane to many small molecules is retained there is a release into the medium of protein derived from the cytoplasmic granules (Woodin, 1961, 1962). When calcium is omitted from the medium the release of the granule proteins is prevented but the cell membrane simultaneously becomes more permeable to the soluble components of the cytoplasm (Woodin & Wieneke, 1963).

It has been suggested that the release of protein may have features in common with the extrusion phase of secretion in mammalian cells (Woodin, 1963). In the leucocyte the process is completed within 10 min. and is not accompanied by regeneration of the granules, so that the biochemical changes associated with protein extrusion are more easily followed. To aid the interpretation of the biochemical changes a study has been made of the morphology of the leucocidin-treated leucocyte and is reported in this paper.

METHODS

Materials. The preparation of leucocidin, polymorphonuclear leucocytes (referred to in this paper as leucocytes) and media for suspending the cells are described by Woodin (1961).

Effect of calcium on the appearance of the leucocidin-treated leucocyte observed with the light-microscope. Two suspensions of 3×10^7 leucocytes in 5 ml. of calcium-free Hanks medium were incubated with leucocidin at 37° for 10 min. and cooled to 0°. To one suspension calcium (75 μl. of 0·11 M-$CaCl_2$) was added and both suspensions were kept at 0°. Samples were placed on slides containing wells in Parafilm (Gladstone, Mudd, Hochstein & Lenhart, 1962) and were observed without a coverslip at ×200 magnification under dark-ground illumination. After 30 min. at 0° samples were also observed under a coverslip at ×900 magnification with dark phase-contrast illumination.

Preparation of material for examination in the electron microscope. Suspensions of 10^7 leucocytes were incubated alone or with leucocidin in 2 ml. of Hanks medium or calcium-free Hanks solution for various periods and cooled in ice. An equal volume of 2% (w/v) osmium tetroxide in sucrose–veronal buffer (Caulfield, 1957) or 0·6% $KMnO_4$ in 0·05 M-veronal (sodium salt) buffer, pH 7·2, was added and the suspensions were maintained at 0° for 1 hr. The suspensions were centrifuged at 60*g* for 3 min. and the supernatant was rejected. The pellet was dehydrated and embedded in Araldite.

The granule fractions of normal and leucocidin-treated leucocytes were isolated by the method of Cohn & Hirsch (1960) from 5×10^8 cells that had been incubated in 10 ml. of Hanks medium for 10 min. A sample of the pellet obtained by centrifuging the granule fraction suspended in 0·32 M-sucrose at 30 000*g* for 30 min. at 0° was fixed in 3 ml. of 1% osmium tetroxide in sucrose–veronal buffer at 0° for 1 hr. and then dehydrated and embedded in Araldite.

To demonstrate the penetration of ferritin or colloidal gold from the medium into the vesicles of the leucocidin-treated cell, suspensions of 5×10^8 leucocytes in 5 ml. of Hanks medium were incubated with leucocidin for 1 min. A portion (1 ml.) of a solution containing 100 mg. of

* Present address: Yale University Medical School, New Haven, Conn., U.S.A.

ferritin in 0·9% NaCl (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) or 12 mg. of colloidal gold chloride in 0·9% NaCl (inactive gold chloride, The Radiochemical Centre, Amersham, Bucks.) was then added and incubation continued at 37° for a further 9 min. The cell suspensions were diluted with 250 ml. of ice-cold Hanks medium and centrifuged at 2000*g* for 5 min. The cell pellets were washed twice in the same way and the granule fraction was isolated from the cell pellets and fixed in osmium tetroxide. In the section of the vesicles derived from the cells incubated with gold, 96 vesicles were examined and the percentage containing gold was determined; 68 vesicles derived from the cells incubated with ferritin were examined in the same way.

Material was stained with phosphotungstic acid by immersing the fixed dehydrated material in an ethanolic 1% phosphotungstic acid solution for 1 hr. before embedding. Staining with lead was done by floating sections on a solution of lead tartrate (Millonig, 1961).

Sections were cut on a Huxley microtome and mounted on Formvar-coated or plain copper grids. They were examined and photographed with a Philips electron microscope 100B at 40 or 60 kv.

Determination of the specific activity of enzymes of the granule fraction of the leucocyte. Two suspensions of 10^9 leucocytes in 35 ml. of Hanks medium, one containing added leucocidin, were incubated at 37° for 10 min. The suspensions were centrifuged at 2000*g* for 3 min. at 0° and the granule fractions from the cell pellets were then isolated. The method of Cohn & Hirsch (1960) was followed, except that the removal of the 'nuclear fraction' by centrifuging was carried out for 20 min. to ensure that any non-granule particulate matter was also removed. The granule fractions were suspended in 10 ml. of 0·34M-sucrose and samples taken for the determination of nitrogen in the material insoluble in 5% (w/v) trichloroacetic acid and the enzyme activities. The acid phosphatase was determined by the method of Cohn & Hirsch (1960): 1 enzyme unit liberates 1 μg. of phosphorus/hr. The assay and unit of activity of peroxidase is given by Woodin (1962). The assays and units of activity of β-glucuronidase and aldolase are described by Woodin & Wieneke (1963).

RESULTS

Light-microscopy. The rapid loss of the streaming motion of the granules and the transition to random brownian motion when the leucocyte is incubated with leucocidin was reported by Gladstone & van Heyningen (1957). The same effect is observed in cells treated with leucocidin in calcium-free media. Under these conditions a few granules can be seen outside the cell. These are not seen when calcium is present and it appears that treatment of leucocytes with leucocidin in calcium-free media can lead to total disruption of a few cells. The increased fragility of the leucocidin-treated leucocyte in calcium-free media has been reported by Woodin & Wieneke (1963).

When leucocytes, previously incubated at 37° with leucocidin in calcium-free Hanks medium, were maintained at 0° in calcium-free Hanks medium the random brownian motion of the granules was maintained for at least 30 min. Phase-contrast microscopy showed that the move ment was not restricted to the periphery of the cell. When calcium was added at 0° to the leucocytes (previously incubated with leucocidin at 37°) the brownian motion stopped and after 15 min. at 0° most of the granules were situated at the periphery of the cell.

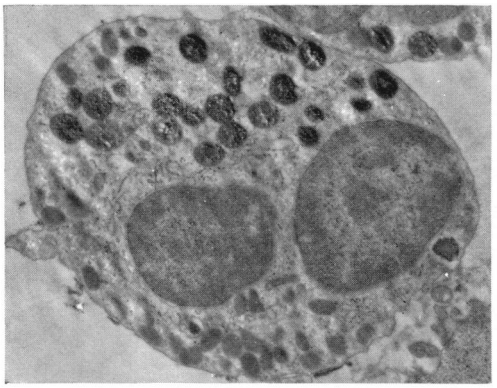
Electron microscopy of leucocytes. Isolated normal leucocytes, fixed with osmium tetroxide after incubation in Hanks medium, did not show marked differences from cells fixed *in situ* (Florey & Grant, 1961). When the sections were stained with lead a reticular network could be seen in the cytoplasm (Plate 1*a*). The granules of normal leucocytes fixed with osmium tetroxide did not show a sharply defined limiting membrane, but this was readily seen in cells fixed with permanganate. This fixative does not stain the granule contents as strongly as osmium and some of the granules do not stain at all (Plate 1*b*).

Plate 1 (*c*) shows a group of leucocytes fixed with osmium tetroxide after incubation for 3 min. at 37° with leucocidin in Hanks solution. The cells were not washed before fixation and hence the cell suspensions contained the protein extruded from the granules. In a separate experiment it was observed that addition of an equal volume of 2% (w/v) osmium tetroxide in veronal–sucrose buffer to the supernatant of leucocidin-treated cells produced a precipitate in a few minutes, and this precipitate became brown after 1 hr. Plate 1 (*c*) shows amorphous extracellular material and it is probable that this is the precipitate of the extruded protein. Most of the cells are degranulated. There are several vesicles in the cytoplasm; some contain amorphous material similar to that outside the cell and some have patches of dense material similar to that of the intact granules. In some cases the surface of the vesicles is continuous with the surface of the cell. Vesicles apparently identical with those in the cytoplasm can be seen outside the cell. The

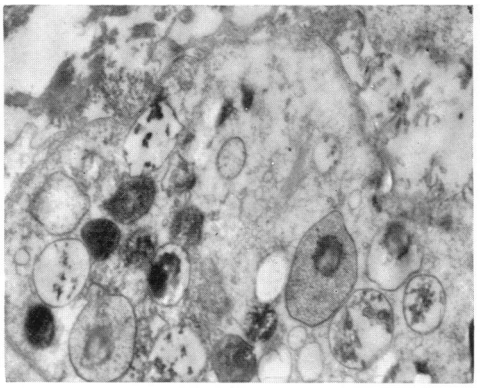
EXPLANATION OF PLATE 1

PLATE 1. (*a*) Normal leucocytes after incubation in Hanks medium. Fixed with osmium and stained with lead. Magnification, ×9 500. (*b*) Normal leucocytes after incubation in Hanks medium. Fixed with permanganate. Magnification, ×7 500. (*c*) Leucocidin-treated leucocytes after incubation in Hanks medium. Fixed with osmium and stained with phosphotungstic acid. Magnification, ×8000. (*d*) Intracellular vesicles of the leucocidin-treated leucocyte. Cells were incubated in Hanks medium, fixed with osmium and stained with phosphotungstic acid. Magnification, ×10 000. (*e*) Leucocidin-treated leucocytes after incubation in Hanks medium. Fixed with permanganate. Magnification, ×9 500.

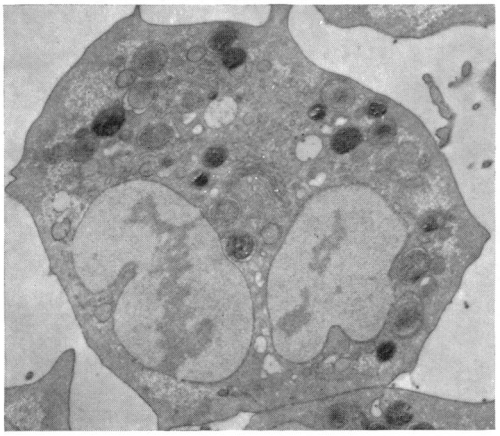
(*a*)



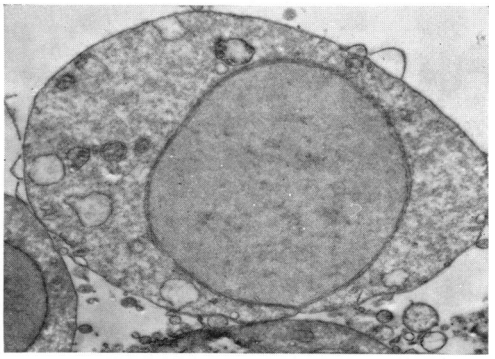
(*d*)



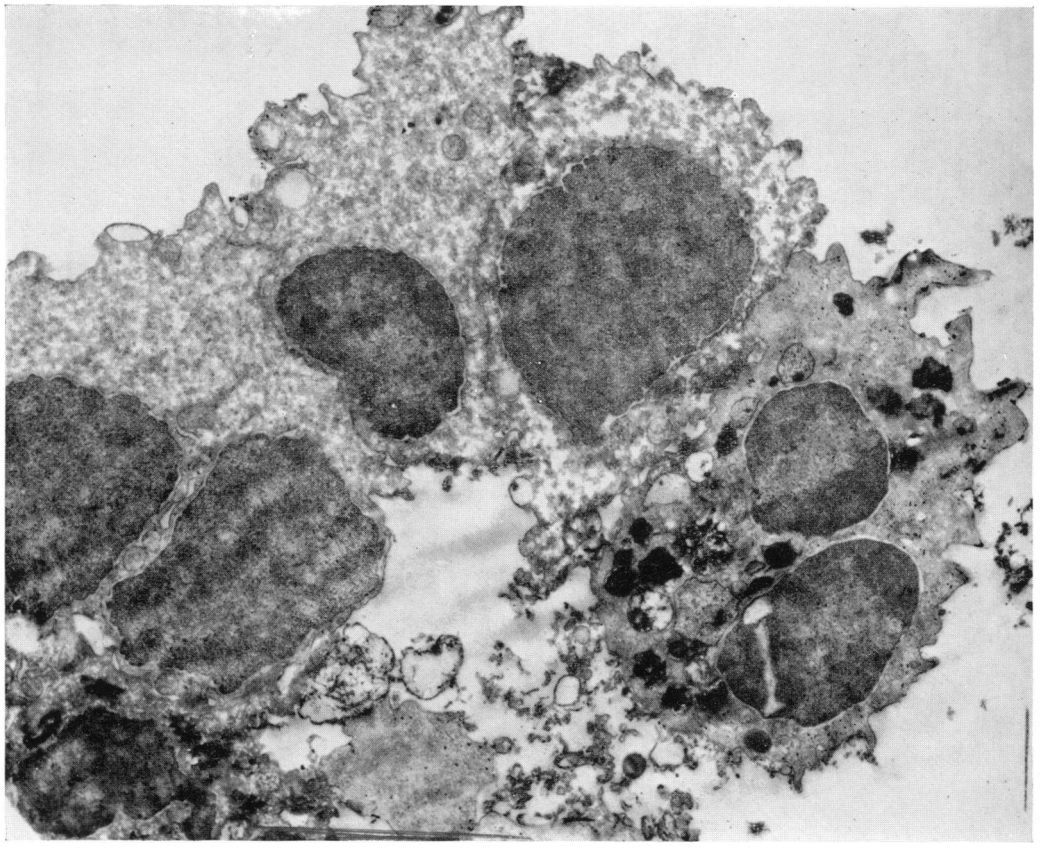
(*b*)



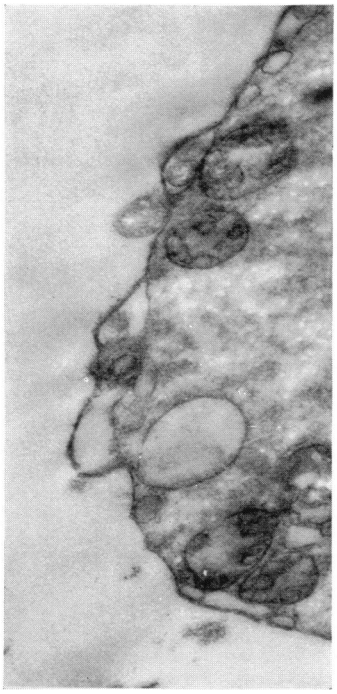
(*e*)



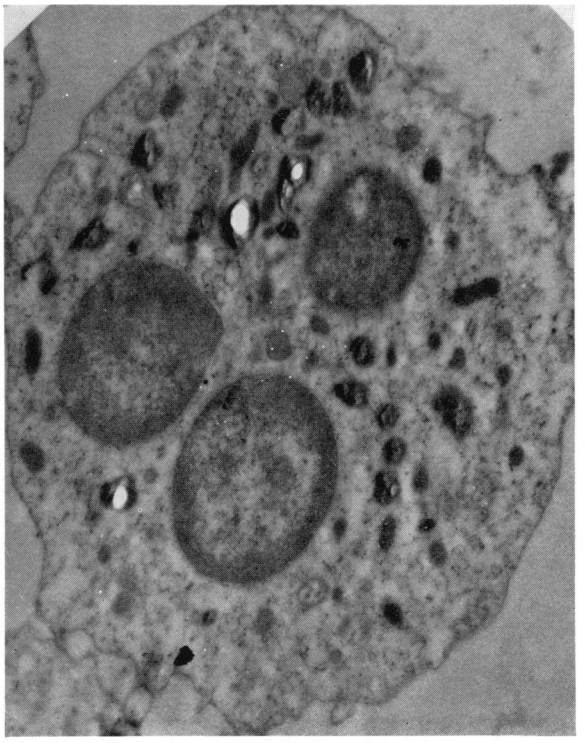
(*c*)



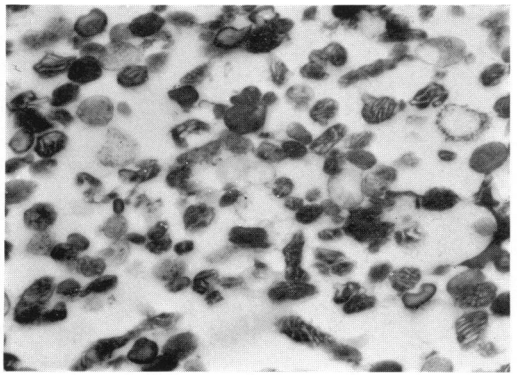
(*f*)



(*g*)



(*h*)



(*i*)

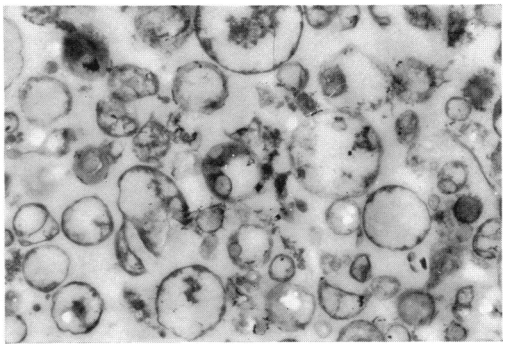


Table 1. *Specific activity of some enzymes of the granule fractions of normal and leucocidin-treated leucocytes*

The granule fractions were isolated by differential centrifuging of homogenates of normal and leucocidin-treated cells that had been incubated in Hanks solution. They were suspended in sucrose and the N content insoluble in trichloroacetic acid and the activities of the enzymes were determined. Specific activities are enzyme units/μg. of N in material insoluble in trichloroacetic acid.

	Sp. activity		
	Acid phosphatase	β-Glucuronidase	Peroxidase
Granule fraction from normal cells	0·21	0·38	0·39
Granule fraction from leucocidin-treated cells	0·28	0·16	0·20

surface of the cell shows many indentations with adhering amorphous material and in places patches of dense material similar to that of the intact granule. The structure of the cytoplasmic vesicles of the leucocidin-treated cell fixed with osmium and stained with phosphotungstic acid is shown in Plate 1 (*d*). Sections of the leucocidin-treated cells stained with lead showed a granular material in the cytoplasm but the reticular network of the normal cell is absent.

Leucocidin-treated leucocytes fixed with osmium after incubation for only 1 min. showed more granules close to the surface than can be seen in normal cells, but continuity between an osmophilic granule and the surface of the cell has not been seen. Apparent continuity of the vesicles and the cell surface is observed in preparations fixed in permanganate (Plate 1*e* and Plate 2*f*). It has been recorded above that the granules of normal cells do not stain intensely with permanganate; similarly the amorphous material extruded from the leucocidin-treated cell is not so prominent in the preparation fixed with permanganate. This has the effect of giving greater contrast to the surface of the cell and the vesicles.

Plate 2 (*g*) shows a leucocidin-treated leucocyte after incubation for 10 min. at 37° in calcium-free Hanks solution. The cell contains many granules but few vesicles can be observed. When leucocidin-treated leucocytes were incubated for 5 min. in calcium-free Hanks solution and then for 5 min. in Hanks solution containing calcium, numerous vesicles were present and the cells had lost most of their granules.

Electron microscopy and composition of isolated granule fractions. Plate 2 (*h*) shows the granule fraction isolated from normal cells. The presence of granules of different morphology in the rabbit leucocyte has been recorded by Marchesi (1961) and by Florey & Grant (1961). Plate 2 (*i*) shows the corresponding fraction isolated from leucocidin-treated cells incubated in Hanks solution. Very few intact granules can be seen and the appearance of the isolated vesicles is identical with that of the vesicles present in the cytoplasm of the leucocidin-treated cell.

If the granules were converted into vesicles within the cytoplasm as a preliminary to their attachment to the surface of the cell they should have the same composition as the granules. Table 1 shows that this is not the case. The retention of much of the acid phosphatase within the granule fraction of the leucocidin-treated cell was reported by Woodin (1962). The increased concentration of this enzyme within the vesicles is taken as evidence that they are not produced by pinocytotic uptake of the medium proceeding independently of the extrusion of the contents of the granules. β-Glucuronidase and peroxidase are typical of those enzymes, most of which are transferred from the granules to the exterior of the cell. No aldolase could be found in the vesicles. Since this enzyme is confined to the soluble fraction of the cytoplasm (Woodin & Wieneke, 1963) it is improbable that the vesicles are produced by vesiculation of the cytoplasm.

When ferritin or colloidal gold was included in the medium it penetrated into 5 % of the vesicles.

DISCUSSION

Early in the treatment of the leucocyte with leucocidin there is a transition of granule motion from streaming to random brownian movement.

EXPLANATION OF PLATE 2

PLATE 2. (*f*) Vesicles of the leucocidin-treated leucocyte adherent to and fused with the cell surface membrane. The cells were incubated in Hanks medium and fixed with permanganate. Magnification, ×25 000. (*g*) Leucocidin-treated leucocyte incubated in calcium-free Hanks solution. Fixed with osmium and stained with lead. Magnification, ×11 000. (*h*) Granules in the granule fraction from normal leucocytes. Isolated from cells incubated in Hanks medium. The granules were fixed in osmium and stained with phosphotungstic acid. Magnification, ×11 000. (*i*) Vesicles in the granule fraction from leucocidin-treated cells. The granule fraction was isolated from cells incubated in Hanks medium. Vesicles were fixed with osmium and stained with phosphotungstic acid. Magnification, ×11 000.

The fact that the movement of the granules of the normal cell is not random indicates that barriers are present which restrict the amplitude of the movement. The electron micrographs of normal leucocytes stained with lead show that the cytoplasm contains a network of membranous material which is absent from the leucocidin-treated cell. The transition to random movement occurs independently of calcium but the subsequent stage of degranulation occurs only when calcium is present. The electron micrographs provide confirmatory evidence that calcium is necessary for extrusion of protein from the leucocyte. The precise role of calcium is discussed by Woodin & Wieneke (1963).

The electron micrographs of the leucocidin-treated cell show numerous vesicles in the cytoplasm and also outside the cell. The biochemical evidence shows that these are not all produced by vesiculation of the cytoplasm or by pinocytosis of the external medium. The vesicles contain amorphous material similar to that seen outside the cell and also patches of dense material similar to the contents of the intact granules. These features and the qualitative similarity of composition are evidence that the vesicles are derived from granules, some of which have discharged part of their contents. On this basis the vesicles would be analogous to those seen in the acinar cells of the pancreas (Ekholm, Zelander & Edlund, 1962).

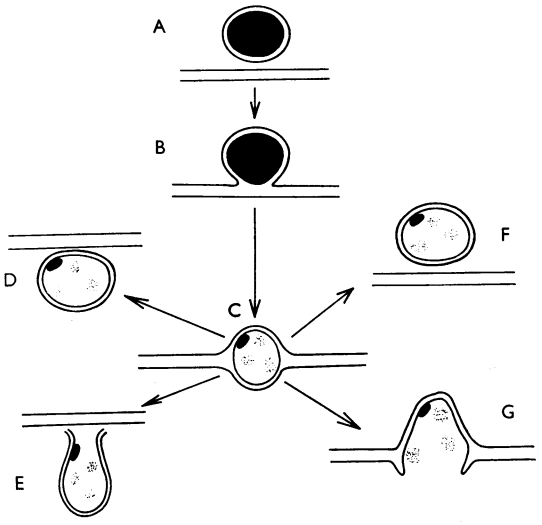


Fig. 1. Hypothetical sequence of events in the degranulation of the leucocidin-treated leucocyte. The top of the Figure represents the interior of the cell, and the bottom represents the medium. The double straight lines represent the cell surface membrane. Spherical black structures represent the granules; the other spherical structures are vesicles. For further description see text.

Fig. 1 summarizes the relationships that can exist between the granules and the vesicles and the cell surface. Stage B is hypothetical; it has not been seen in an electron micrograph. The other situations recorded in Fig. 1 have been frequently seen in electron micrographs of the leucocidin-treated cell. Stage G cannot be distinguished from a situation arising from partial enclosure of external medium by random movements of pseudopodia. The sequence of events suggested by Fig. 1 is purely conjectural; confirmation will not be provided by microscopy of fixed tissues but will require a demonstration of the suggested changes *in vitro*.

The failure to observe fusion of a granule and the cell surface might be because simultaneously with fusion the granule is converted into a vesicle. The conversion of a granule into a vesicle may in part be due to entry of water and swelling, and as the surface of the leucocidin-treated cell is permeable to water (Woodin, 1961) this could occur after fusion of the surfaces of the granule and the cell. A process involving simultaneous fusion of the granule and cell surface and swelling of the granule may contribute to the force required to break the membrane and release the contents to the exterior. There is little doubt that breakage of the membrane after fusion can occur and that this will permit release of proteins, but there is no evidence that this is the only or indeed the major mechanism. The selective permeability of the leucocidin-treated leucocyte (namely, that proteins from the granules but not from the cytoplasm are released, and that the release occurs into the medium but not also into the cytoplasm) could result from an increased permeability occurring only at the site of fusion.

The morphology of extrusion in the leucocyte differs in many respects from that in the pancreas (Palade, 1959; Ekholm *et al.* 1962), the adrenal medulla (de Robertis, Nowinski & Saez, 1960) or the platelet (French & Poole, 1963) and can be related to the special features of the process in the leucocyte. Thus the treatment of leucocytes with leucocidin leads to a loss of control of cation distribution and to cell swelling. The development of a swelling pressure in the leucocidin-treated cell may account for the ejection of some vesicles to the exterior of the cell. Secondly, in the pancreas and adrenal medulla, secretion may proceed continuously and certainly proceeds over several hours whereas in the leucocyte extrusion is confined to a few minutes. The actual extrusion of the proteins in an individual granule may be complete in a few seconds. We have no evidence that the fixatives used in the present work are effective in stabilizing structure so rapidly. Thirdly, the observations on the leuco-

cidin-treated leucocyte are made on cells in free suspension. Under these conditions the localization of the extruded material will not be as effective as in the pancreas or adrenal medulla and the restrictions on the movements of the cell surface of an individual cell due to the resistance to deformation of its neighbours will be absent.

In the pancreas, the adrenal medulla, the platelet and the leucocidin-treated leucocyte, the transfer from the cell of material contained within cytoplasmic particles is associated with fusion of the cytoplasmic particles with the surface of the cell. The subsequent extrusion of the material can be by rupture of the cell surface or by a permeability change at the site of fusion. The relative contribution of these two processes is not known in any secreting tissue.

SUMMARY

1. Leucocytes treated with leucocidin have been examined with the light-microscope and electron microscope.

2. When calcium is present in the medium the cells lose their granules and vesicles can be seen in the cytoplasm and in the medium. Some of the vesicles are fused to the surface of the cell.

3. In the absence of calcium the random brownian motion of the granules persists for at least 30 min. There is no degranulation and vesicles cannot be observed. When calcium is added to the leucocidin-treated cells after incubation in calcium-free medium the brownian motion stops, some of the granules disappear and vesicles can be seen in the cytoplasm.

4. The specific activities of some enzymes in the granules and the vesicles have been measured. It is suggested that the vesicles are produced from granules, some of which have discharged part of their contents. The conversion of the granules into vesicles may be simultaneous with the fusion of the granule and the cell surface.

5. It is suggested that the release of protein from the vesicles follows rupture of the membrane at the site of fusion, or occurs by a permeability change at the site of fusion or by both of these processes.

A.M.W. is a Member of the Scientific Staff of the Medical Research Council.

REFERENCES

Caulfield, J. B. (1957). *J. biophys. biochem. Cytol.* **3**, 827.
Cohn, Z. A. & Hirsch, J. G. (1960). *J. exp. Med.* **112**, 983.
de Robertis, E. D. P., Nowinski, W. W. & Saez, F. A. (1960). *General Cytology*, p. 488. Philadelphia: W. B. Saunders Co.
Ekholm, R., Zelander, R. T. & Edlund, Y. (1962). *J. Ultrastruct. Res.* **7**, 61.
Florey, H. W. & Grant, L. H. (1961). *J. Path. Bact.* **82**, 13.
French, J. E. & Poole, J. C. F. (1963). *Proc. Roy. Soc.* B, **157**, 170.
Gladstone, G. P., Mudd, S., Hochstein, H. O. & Lenhart, N. (1962). *Brit. J. exp. Path.* **43**, 295.
Gladstone, G. P. & van Heyningen, W. E. (1957). *Brit. J. exp. Path.* **38**, 123.
Marchesi, V. T. (1961). *Ciba Found. Study Group: Biological Activity of the Leucocyte*, **10**, p. 76.
Millonig, G. (1961). *J. biophys. biochem. Cytol.* **11**, 736.
Palade, G. E. (1959). *Symp. Subcellular Particles, Woods Hole, Mass.* Ed. by Hyashi, T. New York: Ronald Press Co.
Woodin, A. M. (1961). *Biochem. J.* **80**, 562.
Woodin, A. M. (1962). *Biochem. J.* **82**, 9.
Woodin, A. M. (1963). *Symp. biochem. Soc.* **22**, 126.
Woodin, A. M. & Wieneke, A. (1963). *Biochem. J.* **87**, 480.

Biochem. J. (1963) **87**, 571

Isolation of Phosvitin from the Plasma of the Laying Hen

BY P. J. HEALD AND PATRICIA M. McLACHLAN
Twyford Laboratories Ltd., Twyford Abbey Road, London, N.W. 10

(*Received* 19 *December* 1962)

Heald (1962) has shown that phosphorylserine can be isolated from the plasma phosphoproteins of the laying hen and presented evidence suggesting that all of the phosphoprotein phosphorus was present in this form. It was also pointed out that the overall nature of the phosphoproteins of plasma had not been firmly established. Thus, although Mok, Martin & Common (1961) isolated a small quantity of phosvitin from the plasma of the laying hen after treatment with oestrogens, they were apparently unable to do so from the plasma of the untreated laying hen.

Apart from this work there is no clear evidence to show whether the phosphoprotein of the plasma