AN INVESTIGATION BY ELECTRON MICROSCOPY OF THE NUCLEOSIDE PHOSPHATASE ACTIVITY OF AMPHIBIAN AND MAMMALIAN ERYTHROCYTES

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ABSTRACT

Amphibian and mammalian blood was washed in isotonic saline, fixed in glutaraldehyde, and then stained in the ATPase medium of Wachstein and Meisel. The blood cells were subsequently postfixed in osmium tetroxide, embedded in epoxy resins, and studied by electron microscopy. The plasma membranes of amphibian erythrocytes, from the newt *Triturus cristatus* and the frog *Rana esculenta*, were stained after incubation in media containing ATP or ADP as substrates, but were unstained after incubation in media containing AMP or sodium β-glycerophosphate. The addition of 0.001 M ouabain to ATP-containing media did not inhibit the staining of the plasma membranes, but the omission of Mg^{++} ions from the medium inhibited staining. The plasma membranes of rat and rabbit erythrocytes were never stained after incubation in any of the media used.

With biochemical and cytochemical methods, nucleoside phosphatases have been demonstrated on the plasma membranes of several different cell types, including erythrocytes (see Novikoff *et al.*, 1962 review).

Biochemical investigations have shown that membrane-bound adenosine triphosphatases (ATPases) are present in human erythrocyte ghosts which hydrolyse adenosine triphosphate (ATP) and adenosine diphosphate (ADP), but not adenosine monophosphate (AMP), inosine triphosphate (ITP), or pyrophosphate (Post *et al.*, 1960; Dunham and Glynn, 1961). One of these ATPases is dependent upon Mg^{++} ions and is further stimulated by Na^+ and K^+ ions. This Na^+ and K^+ ion stimulation is inhibited by ouabain, but the basic Mg^{++} ion-dependent activity is not. The Na^+ and K^+ ion-stimulated ATPase is believed to be involved in the active transport of cations (Post *et al.*, 1960; Hoffman, 1960; Dunham and Glynn, 1961; Bonting *et al.*, 1961), and according to Hoffman and Ryan (1960), it is on the inner surface of the erythrocyte plasma membrane. Clarkson and Maizels (1952) and Hoffman and Ryan (1960) state that there is another ATPase activity, which is not stimulated by Na^+ and K^+ ions, on the outer surface of the human erythrocyte plasma membrane.

On the other hand, Garzo, Ullman, and Straub (1952) and Wenkstern and Engelhardt (1955, 1959) found that when erythrocytes from several mammalian species (man, dog, cat, pig, rabbit) were washed and resuspended, unhaemolysed, in isotonic media containing ATP they were unable to hydrolyse the ATP, whereas after haemolysis, the ghosts hydrolysed the ATP either to AMP or to the nucleoside. Garzo *et al.* (1952) found that this activity was associated with the membranes of the erythrocyte ghosts and concluded that mammalian erythrocytes have ATPases on the inner, but not on the outer, surface of the plasma membrane.

Rat erythrocytes with the outer surface of their plasma membranes positively stained have been

observed in material processed for the cytochemical demonstrations of ATPase (Novikoff, 1962; Marchesi and Barrnett, 1963, 1964; and Torack and Barrnett, 1964). In all these reports, however, the stained erythrocytes were in blood vessels; and Novikoff (1962) noted that this staining might be due to a soluble plasma enzyme adsorbed onto the cell surface during fixation. He suggested that this could be tested by studying erythrocytes washed in saline before fixation.

In contrast to their observations on rabbit red cells, Wenkstern and Engelhardt (1955, 59) found that washed but unhaemolysed erythrocytes of pigeon, tortoise, and frog all possess an ATPase activity on the outer surface of the plasma membrane which is not stimulated by Na^+ and K^+ ions; they called this activity ectoapyrase. The pigeon enzyme hydrolysed ATP, ADP, and ITP but not AMP, Na β-glycerophosphate, or pyrophosphate, whereas the tortoise enzyme was less specific and hydrolysed AMP and Na β-glycerophosphate to a small extent. They did not investigate the substrate specificity of the frog erythrocyte ectoapyrase.

In the present experiments, the Wachstein and Meisel (1957) technique was used with electron microscopy to investigate the localisation of the ATPases of amphibian and mammalian erythrocytes. Blood cells from two amphibian species, the newt *Triturus cristatus* and the edible frog *Rana esculenta*, and from two mammalian species, the rat and the rabbit, were used. Washed and unwashed rat and rabbit erythrocytes showed no ATPase activity and were always unstained, whereas both frog and newt erythrocytes have an ATPase activity on the outer surface of their plasma membranes, which can hydrolyse ATP and ADP, but not AMP or Na β-glycerophosphate. In addition, a few observations of the ATPase activities of amphibian leucocytes have been made.

METHODS

The basic procedure used was to wash the blood cells in isotonic saline, briefly fix them in glutaraldehyde, and then incubate them in the ATPase-staining medium. The incubation was followed by postfixation in osmium tetroxide, dehydration, and embedding for electron microscopy.

Fixation and Cytochemical Staining

Blood drawn from the heart of newts and frogs, from the ear vein of rabbits, and from the heart of rats was washed in two changes of ice-cold isotonic sodium chloride solution. The volume of each change of saline was about 100 times that of the blood cells. The buffy coat was not removed.

After the suspension had been centrifuged and the saline solution removed, the blood was fixed for 15 to 20 minutes in an ice-cold 1 or 2 per cent solution of glutaraldehyde in 0.1 M cacodylate buffered at pH 7.2 (Sabatini, Bensch, and Barrnett, 1962). In some experiments, mammalian blood was fixed without washing in a 10- to 20-fold excess of fixative. The fixative was then replaced by ice-cold wash solution (0.1 M cacodylate buffer, pH 7.2, containing 0.25 M sucrose); the cells were washed in 3 to 5 changes of this solution over a period of 10 to 20 minutes. When the suspension had been centrifuged to pellet the blood cells, the last wash solution was removed and the cells were resuspended in the Wachstein and Meisel (1957) ATP-containing incubation medium. The cells were incubated at 25°C for 25 minutes.

In experiments to investigate the substrate specificity of the ATPase, the ATP in the incubation medium was replaced by equimolar amounts of ADP, AMP, or sodium β-glycerophosphate.[1]

Controls, in which cells were incubated in media from which the respective substrate had been omitted, were run with every experiment.

After incubation, the cells were washed 6 to 8 times in 0.25 M sucrose, postfixed for 30 minutes in ice-cold 1 per cent OsO_4 in 0.1 M cacodylate buffer, pH 7.2, containing 0.14 M sucrose, dehydrated, and embedded.

Dehydration, Embedding, and Electron Microscopy

Amphibian blood was processed throughout as a cell suspension. It was dehydrated through a graded series of ethanols (50, 70, 90 per cent, and three changes of absolute ethanol) and then embedded in Epon (Luft, 1961) or Araldite (Glauert and Glauert, 1958). Prior to polymerisation of the embedding medium, the cells were pelleted in gelatin capsules by centrifugation at 10,000 *g*.

This procedure was not adopted with mammalian blood because the smaller cells are much more difficult to pellet when they are suspended in a viscous embedding medium. Instead, after postfixation in OsO_4, the blood was embedded in 2 per cent agar (Davies and Spencer, 1962), and this was then cut into 1-mm cubes which were dehydrated and embedded in Araldite.

In some experiments, samples of amphibian and mammalian bloods were washed in saline, fixed in glutaraldehyde, and then mixed together. Such mixtures were incubated in the ATPase medium and

[1] The ATP, ADP, and AMP were all supplied by the Sigma Chemical Co. Ltd., London, England.

after postfixation in OsO_4 were embedded in 2 per cent agar and then in Araldite.

Thin sections, cut on an A. Huxley microtome fitted with a glass or diamond knife, were mounted on carbon- and collodion-coated grids and examined in a Zeiss E.M.9 electron microscope. Micrographs were taken at magnifications of 1,700 to 20,000 times. Prior to viewing, some of the sections were stained at room temperature in 2 per cent aqueous uranyl acetate solutions for 4 hours.

OBSERVATIONS

On Amphibian Blood

Despite the brief initial fixation in glutaraldehyde followed by incubation at 25°C, the quality of morphological preservation was sufficiently good that all the organelles present in both the red and white blood cells could be readily identified. However, the nuclear envelopes of many erythrocytes showed large gaps which are artefacts presumably caused by nuclear shrinkage (see Figs. 1 and 2).

A layer of electron-opaque granular material—the staining reaction product, lead phosphate—was seen on the outer surface of the plasma membranes of both newt and frog erythrocytes after incubation in media containing the substrate ATP or ADP (see Figs. 2 to 5). The amount of reaction product varied from cell to cell (see Figs. 4 and 5). In any one section, the plasma membranes of most erythrocytes were stained, and typically the lead phosphate covered the whole membrane of each stained cell. The few unstained cells present were scattered irregularly throughout the sections. The reaction product was never associated with the mitochondria or with the nucleus (see Figs. 5 and 2). The dense granules within the mitochondria (see Fig. 5) are not ATPase staining reaction product since they occur in control preparations. Peachey (1964) has suggested that they are accumulations of divalent cations.

The plasma membranes of erythrocytes incubated in the control media were not stained (see Fig. 1), but after incubation in both control and substrate-containing media sparsely scattered small electron-opaque particles (*P*) were visible in the cells (see Fig. 4). These particles are interpreted as a non-specific precipitate resulting from the reaction of the lead ions in the media with anions (*e.g.*, phosphate and sulphate) in the cells.

The presence of the reaction product on the plasma membrane was the only morphological difference between the experimental and control erythrocytes.

Newt erythrocytes incubated in media containing ATP or ADP but without Mg^{++} ions were unstained. As expected, however, the addition of 0.001 M ouabain to ATP- and ADP-containing media had no effect on the staining reaction. The plasma membranes of frog and newt erythrocytes incubated in media containing either AMP or Na β-glycerophosphate instead of ATP were unstained.

Since whole blood was used in these experiments, leucocytes were occasionally seen in the sections. The plasma membranes of basophilic granulocytes were stained after incubation in media containing either ATP or ADP, and were unstained in the corresponding control experiments; staining was most intense on parts of the cell surface thrown into processes and projections (see Fig. 7). The plasma membranes of the occasional neutrophilic granulocytes and lymphocytes seen in these preparations were unstained, even when they were thrown into processes and projections. These limited observations show that amphibian basophilic granulocytes have an ATPase activity on the outer surface of their plasma membranes.

On Mammalian Blood

The plasma membranes of both rat and rabbit erythrocytes failed to stain in these experiments. To ensure that this absence of staining was not due to some fault in experimental procedure, and to allow direct comparison of the staining of amphibian and mammalian erythrocytes under identical conditions, newt and rat or rabbit bloods were mixed and processed together. The plasma membranes of the mammalian erythrocytes were never stained whereas the plasma membranes of the newt red cells, as expected, were stained after incubation in media containing ADP and ATP (see Figs. 4 and 6).

Even when mammalian blood was fixed, without prior washing in saline, in a 10- to 20-fold excess of fixative and then incubated in ATPase media, the plasma membranes of the erythrocytes were always unstained.

DISCUSSION

In these experiments, the complete absence of an electron-opaque layer on the plasma membranes of control erythrocytes and its precise localisation

on the membranes of stained cells support the view that true enzymic activity is being observed.

Freiman and Kaplan (1960) have suggested that the Wachstein and Meisel technique is only capable of staining non-specific polyphosphatases. However, Wachstein *et al.* (1960) and Novikoff *et al.* (1961) state that ATPases can be stained by this method, although Novikoff *et al.* (1961, 1962) have noted that it is difficult to distinguish between ATPases and less specific phosphatases.

Newt and frog erythrocytes, at pH 7.2, hydrolysed ATP and ADP, but not AMP or sodium β-glycerophosphate. This indicates that neither alkaline phosphatase nor 5′-nucleosidase is present, but that either apyrase or ATPase, or ATPase and ADPase or a combination of these three enzymes must be present.

As expected from the work of Novikoff *et al.* (1961), the amphibian erythrocyte ATPase activity demonstrated in these experiments was insensitive to ouabain inhibition. This activity was, however, dependent upon Mg^{++} ion activation.

The occurrence of adjacent stained and unstained amphibian erythrocytes means either that the ATPase activity of some cells is especially susceptible to inactivation by the fixative or, more probably, that it is only intermittently active depending on some specific function of the membrane. Epstein and Holt (1963) observed similar variable staining of the plasma membrane of HeLa cells incubated in the Wachstein and Meisel medium and interpreted their results in terms of local variations in ATPase activity. In HeLa cells the microvilli often stained heavily, whereas the smooth areas of the plasma membrane tended not to stain. The plasma membranes of basophilic granulocytes of the newt also showed this pattern of staining; the plasma membranes of erythrocytes, both staining and non-staining, were invariably smooth and regular, however.

The ATPases known to be present in mitochondria and the endoplasmic reticulum can only be demonstrated cytochemically after very brief fixation (Wachstein *et al.*, 1960, 1962). Presumably in the present experiments they were inactivated by the fixative.

Under the conditions of fixation and incubation employed, rat and rabbit erythrocytes showed no ATPase activity. The discrepancy between this observation and the observed staining of the outer surface of the plasma membranes of rat erythrocytes in blood vessels in tissue sections (Novikoff, 1962; Marchesi and Barrnett, 1963, 1964; Torack and Barrnett, 1964) must be due to differences in experimental conditions. One explanation is that soluble plasma enzyme is adsorbed onto red cells when they are fixed within blood vessels and that this does not occur when blood, with or without prior washing in saline, is suspended in a large excess of fixative. This suggestion, first proposed by Novikoff (1962), is in accord with the results of biochemical studies of saline-washed mammalian erythrocytes made by Garzo *et al.* (1952) and Wenkstern and Engelhardt (1955, 1959).

An alternative explanation is that ATPases on the outer surface of the plasma membranes of rat erythrocytes are completely inactivated when blood is suspended in excess fixative, but not when erythrocytes are fixed in tissue sections or blocks.

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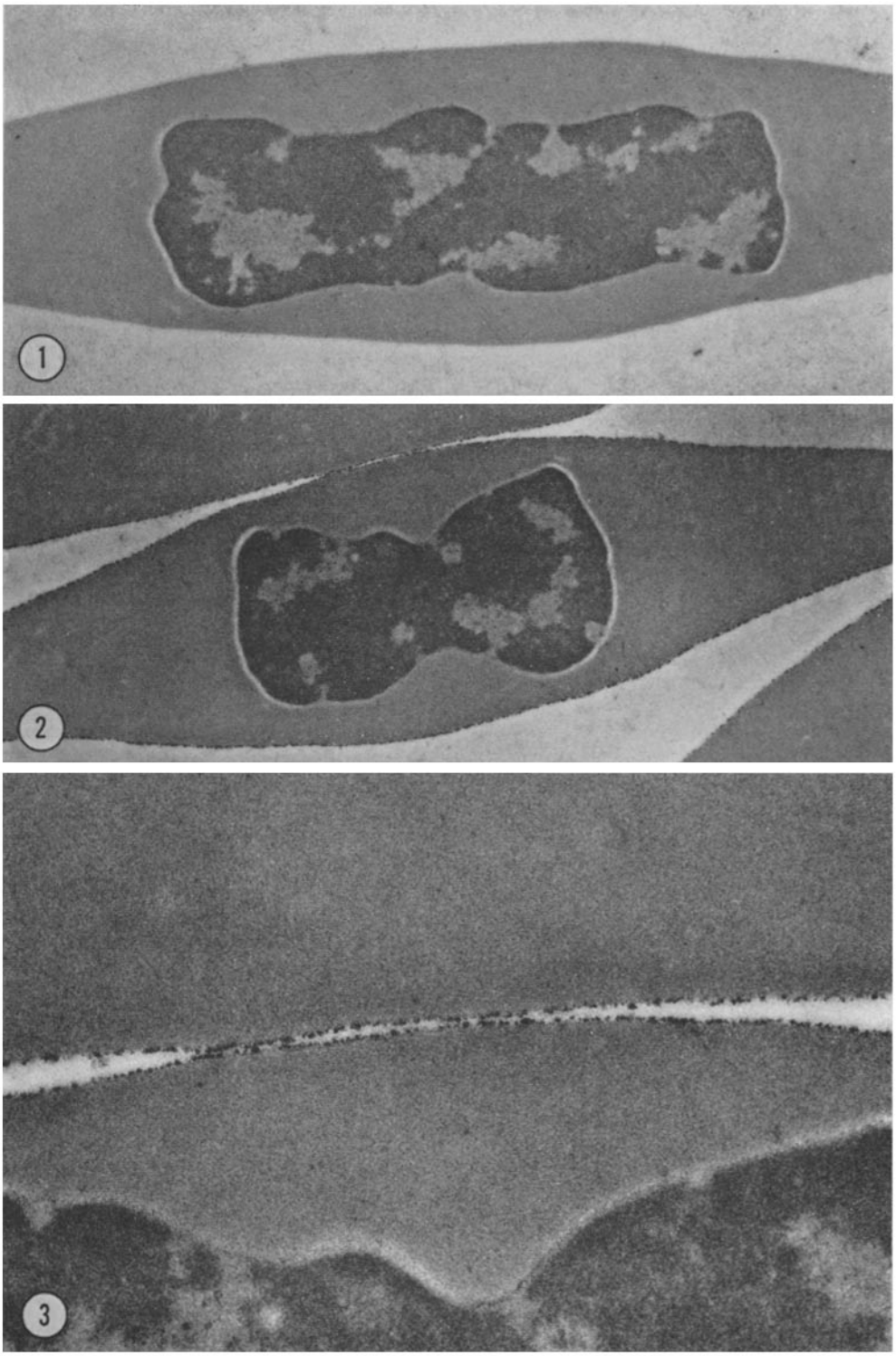
FIGURE 1 A frog erythrocyte incubated in the control medium. The plasma membrane is unstained. Araldite embedded, counterstained with uranyl acetate. × 14,000.

FIGURE 2 A frog erythrocyte incubated in a medium containing ATP as substrate. The plasma membrane is stained; the electron opaque reaction product is restricted to the outer surface of the plasma membrane. Araldite embedded, counterstained with uranyl acetate. × 11,000.

FIGURE 3 An enlargement of a region of the cell in Fig. 2 to show the stained plasma membrane. × 33,000



REFERENCES

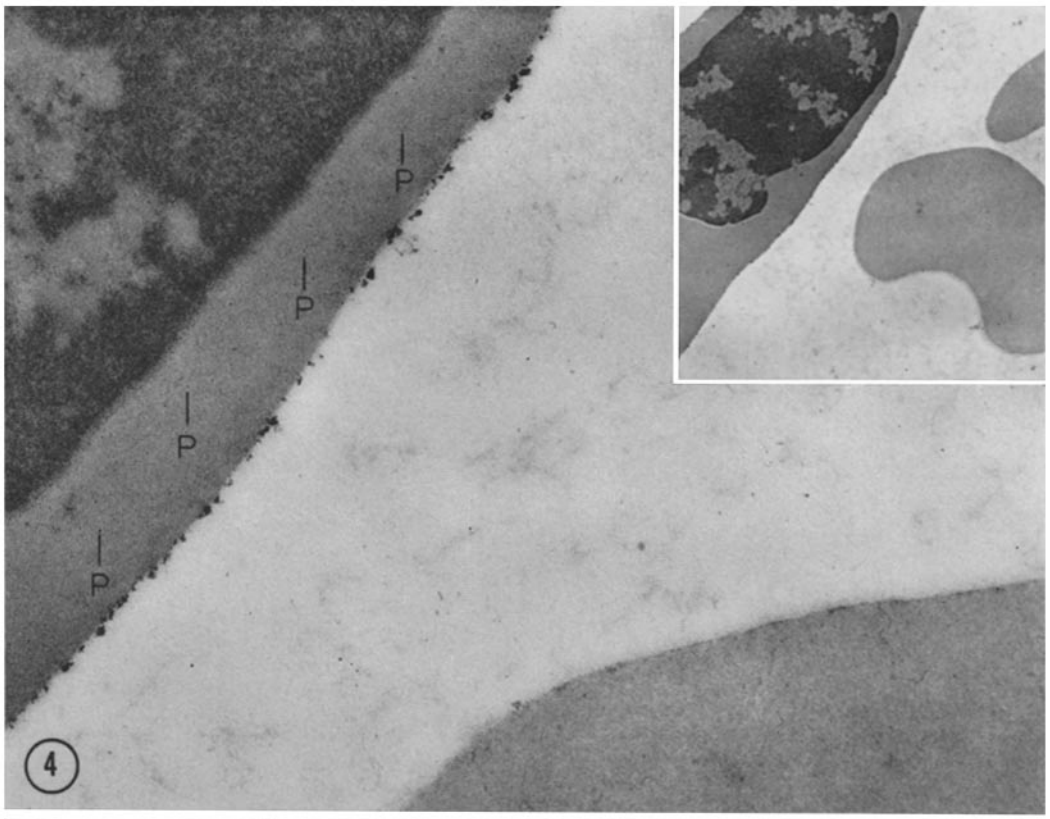
1. BONTING, S. L., SIMON, K. A., and HAWKINS, N. M., Studies on sodium-potassium activated adenosine triphosphatase. 1. Quantitative distribution in several tissues of the cat, *Arch. Biochem. and Biophys.*, 1961, **95**, 416.
2. CLARKSON, E. M., and MAIZELS, M., Distribution of phosphatase in human erythrocytes, *J. Physiol.*, 1952, **116**, 112.
3. DAVIES, H. G., and SPENCER, M., The variation in the structure of erythrocyte nuclei with fixation, *J. Cell Biol.*, 1962, **14**, 445.
4. DUNHAM, E. J., and GLYNN, I. M., Adenosine triphosphatase activity and the active movement of alkali metal ions, *J. Physiol.*, 1961, **156**, 274.
5. EPSTEIN, M. A., and HOLT, S. J., The localization by electron microscopy of HeLa cell surface enzymes splitting adenosine triphosphate, *J. Cell Biol.*, 1963, **19**, 325.
6. FREIMAN, D. G., and KAPLAN, N., Studies on the histochemical differentiation of enzymes hydrolysing adenosine triphosphate, *J. Histochem. and Cytochem.*, 1960, **8**, 159.
7. GARZO, T., ULLMAN, A., and STRAUB, F. B., Die Adenosintriphosphatase der Roten Blutkörperchen, *Acta Physiol. Acad. Sc. Hung.*, 1952, **3**, 513.
8. GLAUERT, A. M., and GLAUERT, R. H., Araldite as an embedding medium for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 191.
9. HOFFMAN, J. F., The link between metabolism and active transport of sodium ions in human red cell ghosts, *Fed. Proc.*, 1960, **19**, 127.
10. HOFFMAN, J. F., and RYAN, H. E., The correlation of ATPase activity with active Na^+ ion transport in human red cell ghosts, Abstracts of the Society of General Physiologists, 1960, (unpublished), quoted in *Circulation*, 1962, **26**, suppl. 1210.
11. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
12. MARCHESI, V. T., and BARRNETT, R. J., The demonstration of enzymic activity in pinocytotic vesicles of blood capillaries with the electron microscope, *J. Cell Biol.*, 1963, **17**, 547.
13. MARCHESI, V. T., and BARRNETT, R. J., The localisation of nucleoside phosphatase activity in different types of small blood vessels, *J. Ultrastruct. Research*, 1964, **10**, 103.
14. NOVIKOFF, A. B., *in* Symposium on the Plasma Membrane, 1126–7, *Circulation*, 1962, **26**, supplement.
15. NOVIKOFF, A. B., DRUCKER, J., SHIN, W-Y., and GOLDFISCHER, S., Further studies of the apparent adenosine triphosphatase activity of cell membranes in formol-calcium-fixed tissues, *J. Histochem. and Cytochem.*, 1961, **9**, 434.
16. NOVIKOFF, A. B., ESSNER, E., GOLDFISCHER, S., and HUES, M., *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor), New York, Academic Press, Inc., 1962, 149.
17. PEARSE, A. G. E., Histochemistry, London, J & A Churchill Ltd., 2nd edition, 1960, 877.

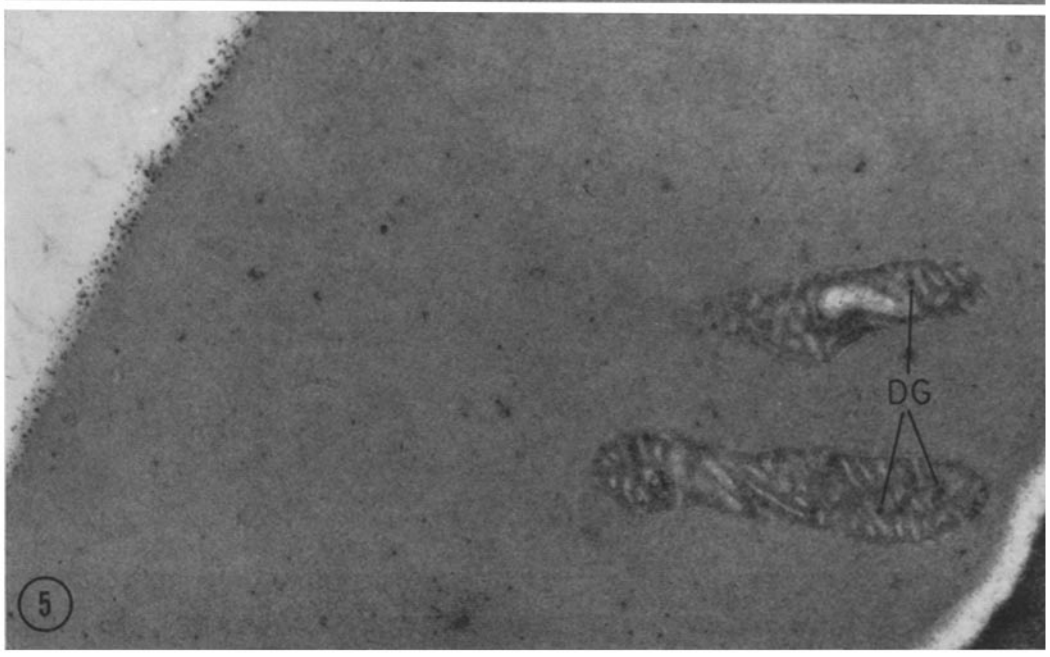
17*a*. PEACHEY, L. D., Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules, *J. Cell. Biol.*, 1964, **20**, 95.

18. POST, R. L., MERRITT, C. R., KINSOLVING, C. R., and ALLRIGHT, C. D., Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in human erythrocyte, *J. Biol. Chem.*, 1960, **235**, 1796.
19. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
20. TORACK, R. M., and BARRNETT, R. J., The fine

FIGURE 4 This micrograph shows newt and rabbit erythrocytes which were washed in saline, fixed, and then incubated together in an ATP-containing medium. The plasma membrane of the newt red cell (left) is stained, whereas the membrane of the rabbit cell is unstained. The sparse fibrous material in the space between the cells is agar. *P*, nonspecific precipitate. Araldite embedded, counterstained with uranyl acetate. × 58,000. The inset shows the two cells at a low magnification. × 6,000.

FIGURE 5 Two mitochondria in a newt erythrocyte incubated in an ADP-containing medium are unstained. The few dense granules (*DG*) in the mitochondria are not stain reaction product. Such granules, a common feature of amphibian mitochondria, occur in control preparations. The plasma membrane, however, is heavily stained. Araldite embedded, counterstained with uranyl acetate. × 30,000.





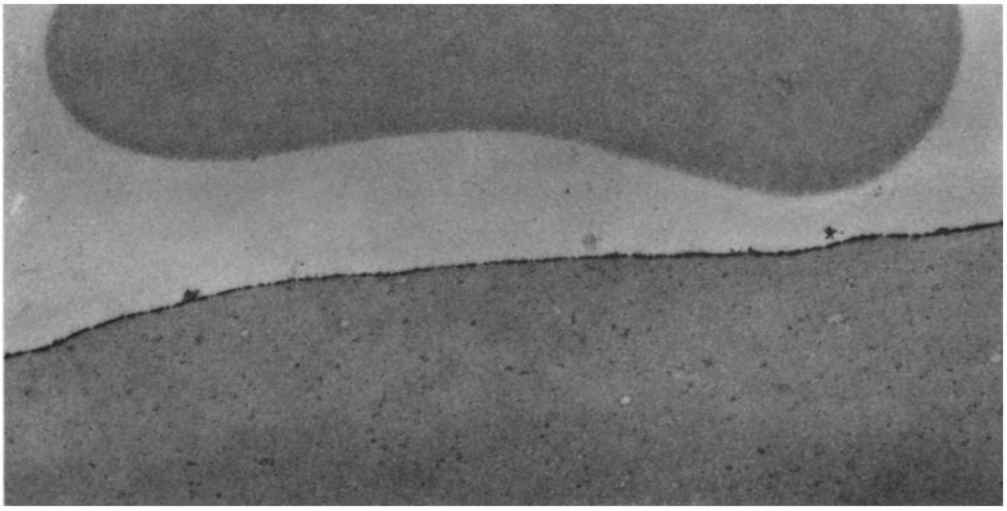


FIGURE 6 This micrograph shows part of a rabbit erythocyte and a newt reticulocyte. The cells were incubated in an ADP-containing medium. Only the plasma membrane of the newt cell (below) is stained. Ribosomal clusters are visible within the newt reticulocyte. Araldite embedded, counterstained in uranyl acetate. × 16,000.

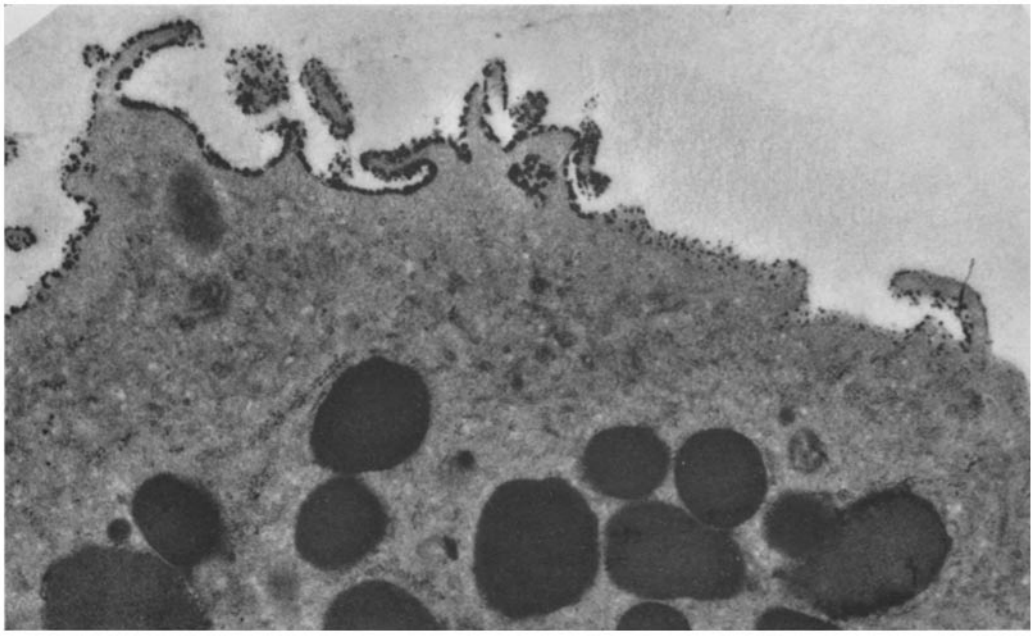


FIGURE 7 Part of a basophilic granulocyte from the newt, which shows staining of the plasma membrane particularly intense in the region with processes, after incubation in an ADP-containing medium. Epon embedded, counterstained in uranyl acetate. × 20,000.

structural localisation of nucleoside phosphatase activity in the blood-brain barrier, *J. Neuropath. and Exp. Neurol.*, **46,** 1964.

21. WACHSTEIN, M., and MEISEL, E., Histochemistry of hepatic phosphatases at a physiologic pH, *Am. J. Chim. Path.*, 1957, **27,** 13.
22. WACHSTEIN, M., MEISEL, E., and NIEDZWIEDZ, A., Histochemical demonstration of mitochondrial adenosine triphosphatase with the lead adenosine triphosphatase technique, *J. Histochem. and Cytochem.*, 1960, **8,** 387.
23. WACHSTEIN, M., BRADSHAW, M., and ORTIZ, J. M., Histochemical demonstration of mitochondrial adenosine triphosphatase activity in tissue sections, *J. Histochem. and Cytochem.*, 1962, **10,** 65.
24. WENKSTERN, T. W., and ENGELHARDT, W. A., Surface localised adenosine polyphosphatase of nucleated erythrocytes, *Doklady Akad. USSR*, 1955, **102,** (1), 133.
25. WENKSTERN, T. W., and ENGELHARDT, W. A., Die an der Oberfläche der Kernhaltiger roten Blutörperchen lokalisierte Adenosinpolyphosphate, *Folia Haematol* (Leipzig), 1959, **76,** 422.