Fine Structural Localization of Adenosine Triphosphatase in Human Platelets and Other Blood Cells

By James G. White and William Krivit

The utilization of adenosine triphosphate (ATP) by human platelets during clot formation and retraction is important to the functioning of these cells in clotting physiology. Recent developments in histochemical and electron microscopic methods have permitted identification of enzymes hydrolyzing ATP at the fine structural level. We have applied these combined technics to the localization of platelet adenosine triphosphatase (ATPase). Enzyme activity was found on platelet surface membranes and in intraplatelet organelles. In the course of this investigation, ATPase activity was also observed in other blood cells. Enzyme reaction product occurred on red cell and lymphocyte surface membranes, and in lymphocyte mitochondria and nucleoli.

Materials and Methods

Preparation of Platelet Rich Plasma

Normal individuals, used repeatedly as controls for coagulation studies in our laboratory, were selected as donors. Venipuncture was performed with siliconized syringes and needles. The blood obtained was transferred immediately to precooled, silicone-coated glass tubes, containing a 2 per cent solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA) in the ratio of 9 parts of blood to 1 part anticoagulant. In several experiments 3.8 per cent sodium citrate was substituted for EDTA, and in other studies no anticoagulant was used.

The samples of whole blood were mixed with the anticoagulant by inversion, and placed in a Servall refrigerated centrifuge at 4°C. Platelet-rich plasma was separated from blood samples after centrifuging at 700 r.p.m. for 15 minutes. The separated samples of platelet-rich plasma were centrifuged at 4°C and 2400 r.p.m. for 30 minutes to obtain platelet buttons. Supernatant plasma was discarded and the samples placed in a refrigerated water bath maintained at 0–4°C. The platelet buttons were washed with cold normal saline (4°C), or 0.08 M cacodylate buffer, pH 7.3, containing 0.18 M sucrose. The cells were washed once, since repeated washings tended to produce cell damage without enhancing the degree of enzyme staining.

Fixation before Enzyme Substrate Incubation

After washing, the platelets were left as buttons, or resuspended in 1 ml. of wash solution. All samples were maintained in the refrigerated water bath throughout the period of processing, except when further sedimentation in the refrigerated centrifuge was required.

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At this time 5 ml. of a cooled (4 C.) fixative was added to the platelet preparation. We have used gluteraldehyde\textsuperscript{14} in 2.5–6.5 per cent concentration, buffered with phosphate or cacodylate buffer to pH 7.3, with or without added sucrose; formaldehyde phosphate;\textsuperscript{15} and hydroxyadipaldehyde,\textsuperscript{14} 3–12.5 per cent in cacodylate buffer, pH 7.3. The several fixative agents, prepared in different concentrations, with different buffers, with or without added sucrose have been used in order to arrive at the combination most suitable for preservation of cell structure and enzyme activity. Although 3 per cent gluteraldehyde in 0.05 M cacodylate had the osmolarity (348 mosm.) closest to that of plasma, it did not prove to be the most effective agent for cell preservation. The most useful agents, applied in the experiments herein reported, have been 6.5 per cent gluteraldehyde in 0.05 M cacodylate buffer, pH 7.3, without sucrose, and 3 per cent hydroxyadipaldehyde in 0.05 M cacodylate buffer, pH 7.3, without added sucrose.

Following the addition of fixative, the platelet buttons, or suspensions, and aldehydes were mixed by gentle swirling. Incubation of the aldehyde-fixed samples was continued for periods of 15 minutes to 24 hours. The shorter periods resulted in cell destruction, and the longer periods in reduced enzyme reactivity. A time of 2 hours in gluteraldehyde, and 4 hours in hydroxyadipaldehyde have proved to be the best compromise.

The aldehyde-fixed platelet preparations were washed three times in the cacodylate-sucrose buffer, or left overnight to wash in the same buffer in order to remove excess aldehyde.

**Enzyme Substrate Incubation**

The Wachstein Meisel media,\textsuperscript{16} as modified by Tice and Barnett,\textsuperscript{17} and Otero de Vilardebo et al.,\textsuperscript{18} were used to localize ATPase activity in the platelet preparations. The Otero-Vilardebo method has consistently given the best results, and was the procedure used for preparation of the cells illustrated in this report. This medium contains ATP 125 mg./100 ml. ($8.3 \times 10^{-4}$M), 2 per cent lead nitrate ($2.5 \times 10^{-3}$M)\textsuperscript{*} and 0.5 M magnesium chloride (0.05 M),* combined in distilled water, and buffered to pH 7.3 with 0.2 M trismaleate (0.08 M).*

The platelet samples were incubated in 5 ml. of the enzyme-substrate media in a 37 C. water bath for 30–90 minutes. Since no enhancement of enzyme localization was observed after 30 minutes, this interval was used in the experiments.

Control samples were prepared by substituting ADP, AMP or sodium $\beta$ glycerophosphate in equimolar concentrations for ATP, or leaving ATP out of the enzyme incubation media.

**Postfixation and Preparation for Electron Microscopic Study**

Following incubation for development of enzyme staining, the cell preparations were washed 3 times in cacodylate sucrose buffer. Five ml. of 1 per cent veranol-buffered osmic acid, pH 7.3, was then added to each sample. Fixation was continued at 4 C. for 1½ hours. The cells were washed in cold veranol buffer, dehydrated in increasing concentrations of acetone, and embedded in vestopal-W.

Thin sections of the plastic-embedded platelet samples were prepared with an LKB ultramicrotome. The sections were examined in either the RCA EMU-3D, or Philips 200 electron microscope. Poststaining of the sections was performed only after initial assessment of unstained material had assured the presence of localized enzyme reaction product.

**Red Blood Cells and Lymphocytes**

Preparation of platelet-rich plasma in the manner described produced a high yield of intact platelets. However, occasional red blood cells and lymphocytes were found.

\*Final concentration.
Fig. 1.—The ultrastructural appearance of human blood platelets. Numerous osmophilic granules (G.) are the predominant intracellular organelles. Only one mitochondrion (*) can be identified in this example. The hyaloplasm is reticular. Occasional vacuoles and vesicles, and particles of glycogen (gly.), are observed. The cell wall (C.M.) has a typical unit membrane structure. Fix.: O.A.; Post Stain: U.A. and L.C. Reduced 35% from Mag. × 61,640.

Enzyme localization in red cells and lymphocytes was noted in cells which were contaminants of the platelet preparations described above, and, therefore, were prepared in an identical manner.

RESULTS

The osmic acid-fixed blood platelet has a characteristic ultrastructural appearance when observed in the electron microscope (fig. 1). Electron-dense granules, together with a few mitochondria and vacuoles, are distributed in the hyaloplasm of the cell and are enclosed by a unit cell membrane. Platelets which have been fixed in the aldehydes before additional fixation in osmium
Fig. 2.—Ultrastructural appearance of platelets initially fixed in gluteraldehyde, incubated in the complete enzyme substrate media for ATPase activity, and post-fixed in osmic acid. Deposits of lead phosphate indicative of ATPase activity coat the surface membrane. The reduced electron density of intracellular organelles is an effect of initial aldehyde fixation. In. Fix.: Glut.; Post Fix.: O.A.; Post Stain: U.A. Reduced 35% from Mag. × 88,570.

have a similar ultrastructure. However, initial fixation, despite repeated washing, apparently prevents the postfixative from producing as high a degree of staining. As a result, the electron density of the unit membranes and the granules is reduced. This defect proves helpful by permitting identification of the enzyme reaction product without confusing it with overstained cell membranes or granules. Addition of uranyl acetate or lead citrate to poststain the sectioned cells corrects the apparent loss of electron density.

Focal deposits of lead phosphate, the reaction product of ATP-ATPase interaction, were found in platelets and other blood cells after incubation in the complete enzyme substrate media. The surface membranes of the plate-
Fig. 3.—An erythrocyte from a preparation similar to that of the platelet in fig. 2. The ATPase activity is indicated by the enzyme reaction product on the cell membrane. In. Fix.: Glut.; Post Fix.: O.A.; Post Stain: U.A. Reduced 35% from Mag. × 31,790.

Platelets (fig. 2), red blood cells, (fig. 3) and lymphocytes (fig. 4) contained deposits of lead phosphate indicative of ATPase activity. The degree of membrane enzyme staining varied considerably. The most reliable and reproducible results occurred with gluteraldehyde-fixed tissue. Cryostat sections of frozen gluteraldehyde-fixed platelets, incubated in the ATP substrate, had more lead phosphate on the cell wall than was observed in the cell membranes of preparations which were not frozen. Internal cell structure was inadequately preserved, however.

Specific intracellular localization of ATPase activity was observed in lymphocytes and platelets. The mitochondria in the cytoplasm of lymphocytes contained the enzyme reaction product (figs. 4 and 5). The more damaged the cell and its mitochondria, the greater the deposition of lead phosphate between mitochondrial cristae. Lead phosphate was also observed in lymphocyte nucleoli when this structure was present in the sectioned cell.
Intracellular localization of ATPase activity in platelets occurred in heavily stained organelles (figs. 6 and 7). Negatively stained membranes in these organelles separating the masses of enzyme reaction product suggested the appearance of mitochondria. However, organelles containing less reaction product could not be differentiated from adjacent unstained granules (fig. 8).

Intracellular enzyme activity was irregularly observed in cells which had been fixed in gluteraldehyde or formal phosphate before enzyme substrate in-
Fig. 5.—Hydroxyadipaldehyde-fixed lymphocyte. Nucelar and cytoplasmic damage is severe, but localization of ATP-ATPase reaction product to mitochondria and cell membrane is specific. The negatively stained leaves of the cristae mitochondriales separate the deposits of lead phosphate. In. Fix.: Hydroxy.; Post Fix.: O.A.; Post Stain: U.A. Reduced 35% from Mag. × 42,500.

cubation. On the other hand, cells which had been fixed in hydroxyadipaldehyde gave reliable evidence of intracellular enzyme, but surface membrane ATPase activity appeared to be decreased. The evident cell damage may have been a factor in the reduced surface membrane ATPase activity of hydroxyadipaldehyde-fixed tissue.
FINE STRUCTURAL LOCALIZATION OF ATP

Fig. 6.—Hydroxyadipaldehyde-fixed platelets. The degree of membrane injury has apparently affected the ATPase activity of the cell wall. Highly specific localization of ATPase is apparent inside the platelets. In. Fix.: Hydroxy.; Post Fix.: O.A.; Post Stain: U.A. Reduced 35% from Mag. × 34,760.

**Discussion**

Hemostasis in man involves a complex interaction of tissue, vascular, plasmatic and cellular components. Among these several factors, the blood platelet appears uniquely endowed to serve a critical role in maintaining hemostatic balance. The mechanisms by which platelets form plugs at the sites of vascular injury, contribute a lipid substance to combine with plasmatic factors in converting prothrombin to thrombin, and act on the platelet fibrin meshwork to
Fig. 7.—High magnification of specifically stained platelet organelles. The membranes around the deposits of enzyme reaction product and narrow leaves within the masses of lead phosphate are negatively stained. Note the similarity of the enzyme-stained platelet organelles to the lymphocyte mitochondria containing enzyme reaction product in fig. 5. The number of enzyme-reacted organelles in platelets is greater than the number of mitochondria ordinarily observed in these cells (fig. 1). In. Fix.: Hydroxy.; Post Fix.: O.A.; Post Stain: U.A. Reduced 30% from Mag. × 182,600.

cause clot retraction have been subjects of intense interest since the work of early investigators.19-23

Recent work has shown that the intermediary metabolism of blood platelets is intimately involved in their function. Platelets have high levels of ATP which are maintained by active enzymatic glycolysis.2 Blockade of this system will
prevent the cells from undergoing sequential alterations in morphology associated with normal function.8-24 The hydrolysis of platelet ATP releases ADP, which causes platelet aggregation.1 A contractile protein with ATPase activity, similar to actomyosin of muscle, has been recovered from platelets.10 This platelet protein may be associated with clot retraction, particularly since ATP appears to be utilized during the process.7 In order to relate the knowledge of platelet function gained by biochemical studies and by morphologic investigations, we have used the combined approach permitted by the development of ultrastructural histochemistry.13

The lead salt method for demonstrating histochemical localization of ATPase activity has been validated by light and electron microscopic studies in a
variety of tissues. Identification of the site of ATPase activity is dependent upon the action of the cell-bound enzyme on ATP of the substrate incubation media. Lead capture ions combine with the terminal phosphate group hydrolyzed from ATP, and the resultant insoluble lead phosphate is deposited at the site of enzyme activity.

The results of this study demonstrate the presence of ATPase activity in the surface membranes of red cells, lymphocytes and platelets. This site of localization is compatible with the results of biochemical investigations which have shown ATPase activity in red cell membranes and in the walls of other tissue cells. ATP-ATPase interaction in surface membranes has been postulated as a mechanism for the transport of ions. The property of ion transport has been demonstrated for the ATP-ATPase complex of the red blood cell membrane. A similar function as a "cation pump" may exist for the membrane ATPase system of lymphocytes and platelets.

Surface membrane ATPase in platelets may also be related to another complex process. Blockade of platelet ATP synthesis by specific inhibitors of enzymatic glycolysis prevents changes in the cell membrane due to pseudopod formation. The necessity of ATP for the morphologic changes in platelet membranes during coagulation suggests that cell wall ATPase may be involved in a contractile protein, ATP-ATPase-dependent system. Contractile proteins have been shown to be important for alterations in surface membranes of primitive cells during cellular division.

The presence of the reaction product of ATP in mitochondria by the technics of this investigation is also compatible with results of biochemical studies. Organelles in the lymphocyte cytoplasm containing enzyme reaction product are mitochondria. Their ultrastructure is apparent despite the obscuring deposits of lead phosphate. The cellular disruption evident in the lymphocytes manifesting mitochondrial ATPase appears to be a necessary factor for penetration of substrate and capture ions into the mitochondria. Lymphocytes which appeared relatively undamaged displayed minimal mitochondrial enzyme staining.

The difficulties surrounding preservation and staining of mitochondrial ATPase have recently been examined. Osmic acid and other fixatives which produce excellent structural appearance of tissues tend to destroy mitochondrial enzymes. As a result, freeze substitution, freeze drying or cryostat sectioning of formalin-fixed frozen tissue have been required to permit mitochondrial ATPase staining by the lead salt methods. The newer aldehyde fixatives introduced by Barnett et al. have been useful in preserving mitochondrial enzymes, but prolonged exposure to the aldehydes inhibits mitochondrial ATPase. Hydroxyadipaldehyde appears to be an exception. Fixation of cell suspensions in this aldehyde for 24 hours before enzyme staining did not prevent the development of mitochondrial ATPase activity in our experiments.

The structure of organelles inside blood platelets heavily stained by the reaction product of ATPase activity was often quite similar to that of enzyme-stained mitochondria of lymphocytes. Negatively stained, narrow membranes separated the deposits of lead phosphate just as did the cristae mitochondriales.
This appearance was not entirely uniform, however, and frequently the ATPase-containing organelles of platelets resembled adjacent unstained granules.

Another problem in deciding the nature of the enzyme-reacted organelles in platelets was due to their number. In 100 thin sections of osmic acid-fixed platelets, we found the number of identifiable mitochondria varied from 0 to 4, with an average number of 2.7 per platelet. The number of ATPase-containing organelles in platelets numbered from 0 to 10, with an average number of 4.2 per cell. Differentiation of granules and mitochondria is often difficult. Previous investigators have pointed out the similarity of platelet granules and mitochondria, and have postulated transitional forms between the two. Others, however, have defined the origin of granules from the golgi complex of the megakaryocyte or its cytomembranes.

On the basis of our studies, it would appear that some granules may have ATPase activity. The structure of platelet granules is far more complex and heterogeneous than has been appreciated, and the possibility that some granules may have a specialized function involving ATP-ATPase metabolism cannot be discounted.

The use of the electron microscope to define fine structural sites of enzyme activity is comparatively new. The rapidly developing field of ultrastructural histochemistry has contributed significantly to our knowledge, but has also created new difficulties in interpretation. These problems have recently been reviewed. The central assumption in such studies, and our own, is that the reaction product appearing on cell membranes and in intracellular sites represents the action of a particular cell enzyme on a specific substrate. In our experiments, the absence of lead phosphate in cells when ATP was omitted from the substrate media, or was replaced by other substrates, supports the view that an ATPase was specifically responsible for lead phosphate deposition when ATP was present. The ATPase activity in mitochondria and cell membranes is further supported by biochemical studies identifying the presence of ATPase at these sites. Novikoff et al. have indicated that the enzyme activity appearing after incubation in the Wachstein-Meisel media with ATP as substrate is in all probability a specific ATPase, rather than a nonspecific form of this enzyme.

Platelet ultrastructural changes and biochemical alterations during clot formation and retraction are intimately bound to energy metabolism in these cells. The fine structural localization of ATPase activity in the unaltered platelet has provided a means of relating morphology and physiology. Similar studies of enzyme localization in the platelet-fibrin meshwork should provide a greater understanding of metabolism and structure in relation to platelet function. Work is currently underway to examine this possibility.

**SUMMARY**

Adenosine triphosphatase activity has been localized in human blood cells by combined histochemical and electron microscopic technics. Deposits of lead phosphate, indicative of enzymatic activity, were observed on the surface membranes of erythrocytes, lymphocytes and platelets. Intracellular ATPase
activity was found in lymphocyte mitochondria and nucleoli. Blood platelets contained organelles in their hyaloplasm with dense accumulations of the enzyme reaction product. The distribution of lead phosphate in platelet organelles suggested that they were mitochondria, but some platelet granules may also contain ATPase activity.

**SUMMARIZIO IN INTERLINGUA**

Le activitate de triphosphatase de adenosina in human cellulas sanguinee esseva localisate per combinate technicas de histochimia e microscopia electronica. Depositos de phosphato de plumbo, indicative de activitate enzymatic, esseva observate in le membranas de superficie de erythrocytos, lymphocytos, e plachettas. Activitate de intracellular triphosphatase de adenosina esseva trovate in mitochondrios e nucleolos lymphocytic. Plachettas sanguinee contineva organellas in lor hyaloplasma con dense accumulationes del producto de reaction enzymatic. Le distribution de phosphato de plumbo in le organellas del plachettas suggestionava que illos esseva mitochondrios, sed certe granulos plachettal etiam contine possibilemente activitate de triphosphatase de adenosina.

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