# THE LOCALIZATION OF Mg–Na–K–ACTIVATED ADENOSINE TRIPHOSPHATASE ON RED CELL GHOST MEMBRANES

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ABSTRACT

The lead salt method introduced by Wachstein and Meisel (12) for the cytochemical demonstration of ATPase activity was modified and used to determine sites of activity on red cell ghost membranes. Preliminary studies showed that aldehyde fixation and standard concentrations of the capture reagent $Pb(NO_3)_2$ resulted in marked inhibition of the ATPase activity of these membranes. By lowering the concentration of $Pb^{2+}$ and incubating unfixed red cell ghosts, over 50% of the total ATPase activity, which included an ouabain-sensitive, Na–K-activated component, could be demonstrated by quantitative biochemical assay. Cytochemical tests, carried out under the same conditions, gave a reaction product localized exclusively along the inner surfaces of the ghost membranes for both Mg–ATPase and Na–K–ATPase. These findings indicate that the ATPase activity of red cell ghosts results in the release of $P_i$ on the inside of the ghost membrane at sites scattered over its inner aspect. There were no deposits of reaction product on the outer surface of the ghost membrane, hence no indication that upon ATP hydrolysis $P_i$ is released outside the ghosts. Nor was there any clear difference in the localization of reaction product of Mg–ATPase as opposed to that of Na–K–ATPase.

## INTRODUCTION

An ATPase[1] activated by $Mg^{2+}$, $Na^+$, and $K^+$, hereafter referred to as Na–K–ATPase, is found associated with cell membranes (1–3) and cell fractions isolated from a wide variety of tissues (4–9). Physiological studies on red cell ghosts and isolated squid axons have provided evidence that this ATPase is either part of or functionally associated with the mechanism of active transport of $Na^+$ and $K^+$ (10, 11). Similarities between the active transport mechanism and this ATPase activity include requirement for ATP and $Mg^{2+}$, activation by $Na^+$ and $K^+$, and specific inhibition of both by the cardiac glycoside, ouabain.

In addition to this Na–K–ATPase, each of the membrane and microsome preparations mentioned exhibits an ATPase activity which is dependent only on $Mg^{2+}$ and is not inhibited by ouabain. This second activity, hereafter referred

[1] The following abbreviations are used in this paper: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate, UTP, uridine triphosphate; $P_i$, inorganic phosphate; SDS, sodium dodecyl sulfate; EDTA, ethylene diaminetetraacetate; DEAE-cellulose, diethyl aminoethyl cellulose; NADH, nicotinamide adenine dinucleotide, reduced; Tris, tris(hydroxymethyl) aminomethane.

to as Mg–ATPase, is presumably not involved in cation transport, but its exact relationship to the Na–K–ATPase is not fully understood: the two activities have been ascribed to two distinct enzymes or, conversely, considered as representing two different functional states of a single enzyme.

Both activities are known to be associated with the red cell membrane but their location in this membrane remains unknown. Previous investigators have attempted to determine sites of ATPase activity on membranes by using the lead-salt precipitation method of Wachstein and Meisel (12). However, objections to these studies have been raised on the grounds that the methods used could not demonstrate Na–K–ATPase since this activity was either destroyed by aldehyde fixation or inhibited by the heavy metal used as capture reagent (13). Other objections have pointed out that, under the conditions of current cytochemical procedures, nonenzymatic hydrolysis of ATP by lead could occur (14).

The present studies were initiated in an attempt to answer the following questions: (*a*) can a cytochemical ATPase procedure be devised free of the objections mentioned? (*b*) can the Na–K–ATPase of red cell membranes be demonstrated, and if so, where is this activity localized on the membrane? (*c*) can the Mg–ATPase be differentiated from the Na–K–ATPase on the basis of difference in localization on the membrane?

We have chosen red cell ghosts as an experimental system since they consist of a single membrane and can be prepared easily, and since their ATPase activity remains stable for relatively long periods. Although compared to other tissues (13) red cells are not particularly active, their ATPase activity is well characterized and their membrane represents a system in which the correlation between active transport and ATPase activity is reasonably secure (1, 2, 10).

For these studies, a standard red cell ghost preparation was made from guinea pig blood and experiments were carried out so that the most favorable conditions could be determined for the cytochemical demonstration of both Mg– and Na–K–ATPases. The responses of the red cell ghost system to prefixation by aldehydes and to the presence of heavy metals used as capture reagents were investigated in light of the reports described above. In addition, in view of data available on the ATPases of other membrane systems (15, 16), the effect of detergents and of the physical state of the ghost membranes upon their ATPase activity was studied.

## MATERIALS AND METHODS

### *Preparation of Red Cell Ghosts*

Blood was obtained by cardiac puncture from adult guinea pigs under $CO_2$ narcosis, and ghosts were prepared according to the following procedure:

whole blood centrifuged 2,500 rpm, 15 min, with 0.1 M $Na_3$ citrate added as anticoagulant (5 ml/40 ml whole blood)

| | |
|---|---|
| Plasma and buffy coat aspirated off | Packed red cells washed twice in 0.9% NaCl, and recovered by centrifugation as above; lysed in 10 vol of 5 mM Tris–HCl, pH 7.0, 1 mM EDTA,[2] (*a*) mixed 15–20 min; centrifuged 25,000 *g*, 30 min; |
| supernatant discarded | red cells resuspended in same medium, mixed, centrifuged 25,000 *g*, 10 min; |
| supernatant discarded | (*b*) red cells suspended in 0.05 M Tris–HCl, pH 7.0, 0.50 M NaCl, 1 mM EDTA,[2] centrifuged 25,000 *g*, 10 min; |
| supernatant discarded | red cells (now faint pink in color) suspended in 5 mM Tris–HCl, pH 7.0, 1 mM EDTA,[2] centrifuged 25,000 *g*, 10 min; |
| supernatant discarded | ghosts suspended in same medium, centrifuged 25,000 *g*, 10 min, |
| supernatant discarded | ghosts (now milky white) suspended in small volume (~0.5 ml/1 ml pellet) of 0.05 M Tris–HCl, pH 7.0. |

(*a*) This and all other operations after the first hemolysis were carried out in the cold. It was necessary to centrifuge the initial hemolysate at 25,000 *g* for 30 min to insure the complete sedimentation of all ghosts from the dense and viscous hemoglobin-containing medium. This high speed was used in subsequent steps to shorten the centrifugation times.

(*b*) Treatment of ghosts with this high salt wash

[2] A 0.1 M stock solution of $Na_2$-EDTA, neutralized to pH 7.0 with 2 M Tris, was used.

was an important step in rendering the ghosts free of most of the residual hemoglobin.

The protein content of the final ghost preparations was determined by the Lowry procedure (17) with bovine serum albumin as the standard.

### *Ultrasonic Treatment of Ghost Membranes*

Ghost preparations were sonicated with a Branson Ultrasonifier for varying periods while kept immersed in an ice bath. Disruption of the ghosts to apparent completion was monitored by phase-contrast microscopy.

### *Materials*

ATP and other nucleotides were purchased from Sigma Chemical Co. (St. Louis, Mo.). The disodium salt of ATP was dissolved in water and neutralized to pH 6.8 with Tris. ATP concentrations in stock solutions were determined spectrophotometrically with the assumption of a molar absorbancy of $15.3 \times 10^3$ at 259 mμ. All other materials used in incubation media were of analytical grade when available.

$^{32}$P-labeled ATP was prepared enzymatically according to the method of Glynn and Chappell (18) and was modified as follows. Under appropriate conditions $^{32}P_i$ was incubated with ATP, 3-phosphoglyceric acid, 3-phosphoglyceric acid kinase, glyceraldehyde 3-phosphate dehydrogenase (all purchased from Sigma Chemical Co.), and NADH. [$\gamma$-$^{32}$P]-ATP was recovered from the $HClO_4$-treated reaction mixture by passing the neutralized supernate through a Sephadex G-10 (Pharmacia Fine Chemicals Inc., New Market, N. J.) column (2.5 × 90 cm) and eluting with 0.05 M Tris–HCl, pH 6.8. This procedure provided a simple, one-step separation of [$^{32}$P]-ATP from $^{32}P_i$. [$^{32}$P]-ATP was assayed by thin layer chromatography using DEAE-cellulose-coated plates and a formic acid solvent as described by Fahn et al. (19). All of the $^{32}$P bound to ATP was located in the $\gamma$-position as determined by a chromatographic analysis of the products of incubation of [$^{32}$P]-ATP with glucose and hexokinase.

1–2% of the radioactivity of the [$^{32}$P]-ATP stock was present as $^{32}P_i$ depending on the preparation and length of storage.

### *ATPase Assay*

CHEMICAL DETERMINATION OF $P_i$: Assay systems for ATP hydrolysis contained Tris–HCl or Tris–maleate buffers, appropriate cations and other agents, substrate, and usually ~1 mg ghost protein in a total volume of 1.0 ml (see figure legends for further details). Incubations were carried out in a 37° water bath–shaker and were terminated by chilling followed by the addition of 0.2 ml of 10% $HClO_4$. All materials were kept cold after this point. After centrifugation, aliquots of the supernate were assayed for $P_i$ by a modified Fiske-Subbarow procedure. The assay system contained 0.2 ml acidified sample, 0.1 ml 2.5% $NH_4$ molybdate, 0.1 ml 1% Elon (Eastman Kodak Co., Rochester, N. Y.) as reducing agent, and water to 1.0-ml volume. The optical density at 660 mμ was linear with $P_i$ concentration over the range used. Heat-treated ghosts (80°, 5 min) were used as controls so that ATP blanks could be determined. All assays were carried out in duplicate.

$^{32}$P RELEASE FROM [$^{32}$P]–ATP: When [$^{32}$P]–ATP was used as substrate, acid-washed Norit "A" (Amend Co., New York) was added to the $HClO_4$-treated reaction mixture, and aliquots of supernate containing nonNorit adsorbable $^{32}$P were dried on planchets and counted in a Nuclear-Chicago Corporation (Des Plaines, Ill.) gas flow counter. Parallel chemical determinations showed that the $^{32}$P of the supernate was in the form of $P_i$.

### *Cytochemical Demonstration of ATPase Activity*

The incubation media contained Tris–maleate buffer, pH 7.0, appropriate cations (added as chlorides), ATP or other substrates, and $Pb(NO_3)_2$ at concentrations given in the legends of the figures; the reagents were pipetted in the above order. The $Pb(NO_3)_2$ was added to the medium while the tube was shaken on a Vortex mixer to prevent precipitation. All cytochemical media were completely clear before the addition of the ghosts. With the exception of $Pb(NO_3)_2$, the composition of the cytochemical medium was identical with that of the biochemical assay and included optimal concentrations of ATP, $Mg^{2+}$, and $Na^+$ and $K^+$.

Reactions were run with unfixed ghosts or ghosts prefixed in glutaraldehyde (20); in the case of unfixed ghosts incubations were carried out at 30° or 37° for varying periods ranging from 10 to 30 min, and the reaction was stopped by chilling the incubation mixture to 4° followed by the addition of two volumes of 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0.

After storage in the cold for several hours (the time could be varied without any noticeable effects), the mixture was centrifuged (40,000 *g*, 30 min), and the pellets were washed in buffer and postfixed in 1% $OsO_4$ in either phosphate or Veronal–acetate buffer (pH 7.0). Most of the pellets were stained in uranyl acetate directly after $OsO_4$ fixation by methods described previously (21). After staining for 2 hr the pellets were dehydrated in ethanol and embedded in Epon. Sections were cut with a Porter-Blum microtome provided with diamond knives. Most sections were further stained with uranyl acetate and lead citrate before examination in a Siemens Elmiskop I electron microscope operated with a 50 μ objective

aperture and an anticontamination device. Micrographs were taken at magnifications ranging from 10,000 to 40,000. Preliminary studies showed that the staining of tissues in block with uranyl acetate or the staining of sections with combined uranyl–lead stains did not change the appearance or location of Pb–phosphate precipitates.

In some experiments, ghosts were fixed in 0.5–2% glutaraldehyde (30 min in the cold room) so that it could be determined whether prefixation affected the localization of reaction products on the membranes. At the end of fixation, the ghosts were recovered by centrifugation and washed in Tris–maleate buffer three times before incubating in the ATPase medium; after incubation, they were processed as described above, with double fixation with glutaraldehyde and $OsO_4$.

## OBSERVATIONS

### *Red Cell Ghosts*

When prepared as described, the red cell ghost suspension is milky white; by phase microscopy, the ghosts appear as empty sacs which in form and size still resemble swollen red cells. When viewed after incubation in a medium containing ATP, the ghosts are shrunken and smaller in size and many assume a biconcave disc shape.

Electron micrographs confirm the impression that the ghosts are essentially empty sacs (Fig. 1). The membranes of individual ghosts are entirely continuous and the usual, layered structure (unit membrane) is seen wherever the section is perpendicular to the membrane plane. In addition, these membranes have filamentous material attached to their inner surfaces which appears more prominent after incubation in a medium containing ATP (Fig. 2). In some sections, the filamentous material appears to be part of a feltlike network attached to the inner leaflet of the membrane. Although its chemical nature in unknown, this filamentous material serves as a useful marker of the inside surface of the ghost membranes.

When ghosts are subjected to relatively short bursts of ultrasound (10–15 sec at the No. 6 setting), they are broken up into small globules. After longer treatment (30–60 sec) the preparation becomes a turbid, cloudlike haze which contains practically no resolvable particles in the phase-contrast microscope. When such preparations are fixed and studied by electron microscopy, the hazy mass is found to consist of minute vesicles which are limited by continuous unit membranes (Fig. 3) indistinguishable from the original ghost membranes: they have the same dimensions and the same filamentous material on their inner surfaces (Fig. 4). Most membranes are organized in closed vesicles; few membrane fragments with free edges are encountered.

### *ATPase Activity of Red Cell Ghosts*

Since the ghosts used in this study were prepared by a new method that employs a strong salt wash, and since the ATPase assay system was modified for possible parallel use as cytochemical reaction medium (these modifications included the use of a Tris–maleate buffer and the presence of heavy metal salts), the following experiments were carried out so that it could be determined whether the properties of our ghost ATPase, under the conditions of our assay, were similar to the previously described properties of ghost ATPase after conventional preparative and assaying procedures.

ATP breakdown by the ghost preparations was found to proceed linearly with time when incubation media contained $Mg^{2+}$, $Mg^{2+}$–$Na^+$–$K^+$, or $Mg^{2+}$–$Na^+$–$K^+$–ouabain (Fig. 5). The rates of ATP hydrolysis in the presence of $Mg^{2+}$ and $Mg^{2+}$–$Na^+$–$K^+$–ouabain were identical, whereas the activity was greater than twofold in the presence of $Mg^{2+}$–$Na^+$–$K^+$ without ouabain. Table I shows that ATP was the substrate most rapidly hydrolyzed by the ghost preparation under the incubation conditions used. The lack of reactivity of ADP suggested that only the terminal phosphate of ATP contributed to the $P_i$ measured by chemical analysis. This was confirmed with data from similar incubations with $\gamma$-$^{32}P$-labeled ATP as substrate.

$P_i$ added to the incubation medium did not exert any inhibitory effect on the ATPase activity of the ghost membranes within the limits tested (up to 4.0 mM). ADP was found to compete with ATP when it was added at relatively high concentrations, but the amount of ADP which was generated by the ghost ATPase in the assay system was well below inhibitory levels. These results indicated that the ATPase activity was not inhibited by the reaction products in our assay system and that the addition of an ATP-generating system was not essential for maximal activity.

### *ATPase Activation by Detergents*

Detergents have been used to modify the ATPase activity of different membrane preparations (15, 22). Skou has reported that the Na–K–ATPase

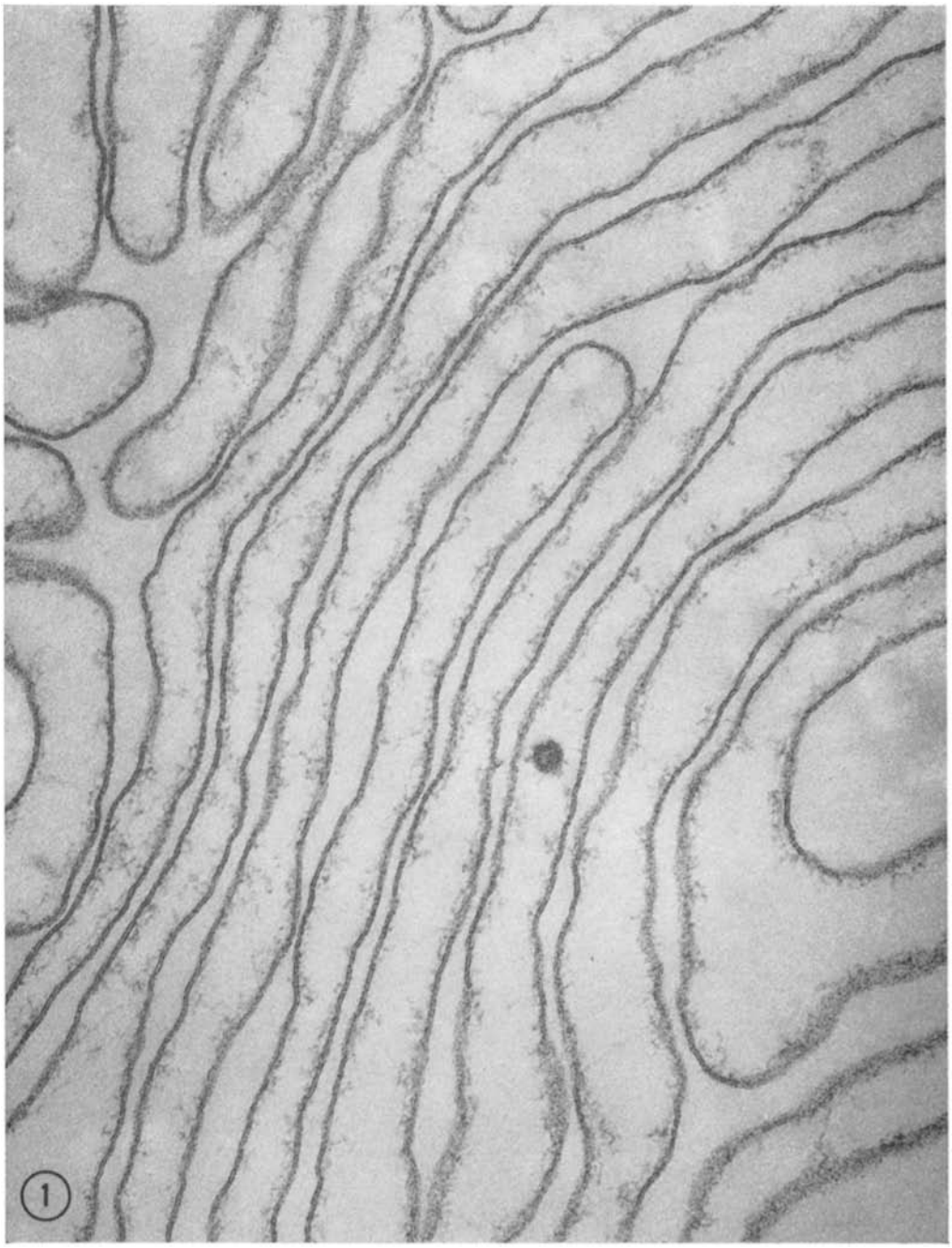
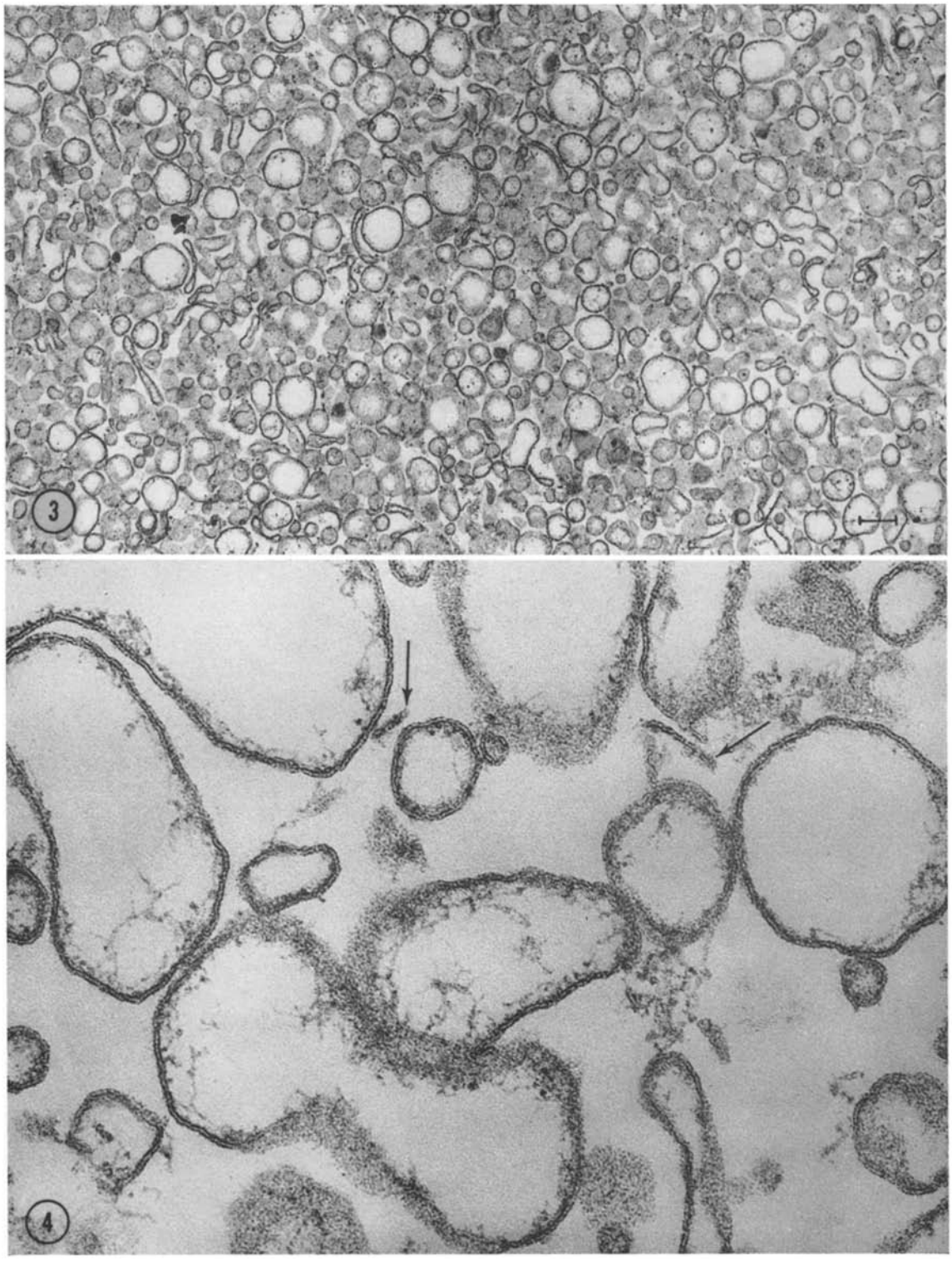



FIGURE 1 Electron micrograph of a representative area in a section through ghost membranes pelleted by high speed centrifugation (100,000 *g*, 30 min) and fixed in glutaraldehyde–$OsO_4$. The ghosts appear as empty sacs bounded by continuous unit membranes. Fibrillar material is seen along the inner surfaces of the ghost membranes. × 90,000.


FIGURE 2 Small field in a red cell ghost preparation pelleted and fixed after incubation in a medium containing ATP, $Mg^{2+}$, $Na^{+}$, and $K^{+}$ (concentrations as given in Table I). The filamentous material attached to the inner surface of the ghost membrane appears more prominent than in nonincubated preparations. × 120,000.

activity of nerve microsomes can be selectively increased by treatment with deoxycholate (15), an effect that has been ascribed to the conversion of Mg–ATPase into Na–K–ATPase.

So that it could be determined whether the ATPase activity of our ghost preparation shows similar responses to detergents, ghosts were treated with varying concentrations of the anionic detergent, sodium dodecyl sulfate (SDS), the cationic detergent, cetyl pryidinium chloride, and the nonionic detergent, saponin. Preincubation of ghosts with either SDS or saponin resulted in an activation of both ATPase activities at low concentrations of detergents, while increasing the detergent concentration eventually resulted in the complete inhibition of both components. Fig. 6



FIGURES 3 and 4 Red cell ghosts were subjected to ultrasound for 45 sec, then centrifuged (100,000 *g*, 30 min), and fixed as a pellet in glutaraldehyde–$OsO_4$. The ghost fragments still form closed vesicles, most of which are below the resolving power of the light microscope. The bar on Fig. 3 measures 0.2 μ. In most cases, filamentous material is confined to the inner surfaces of the closed vesicles (Fig. 4); the arrows mark rare membrane fragments which show free edges. Fig. 3, × 30,000; Fig. 4, × 90,000.

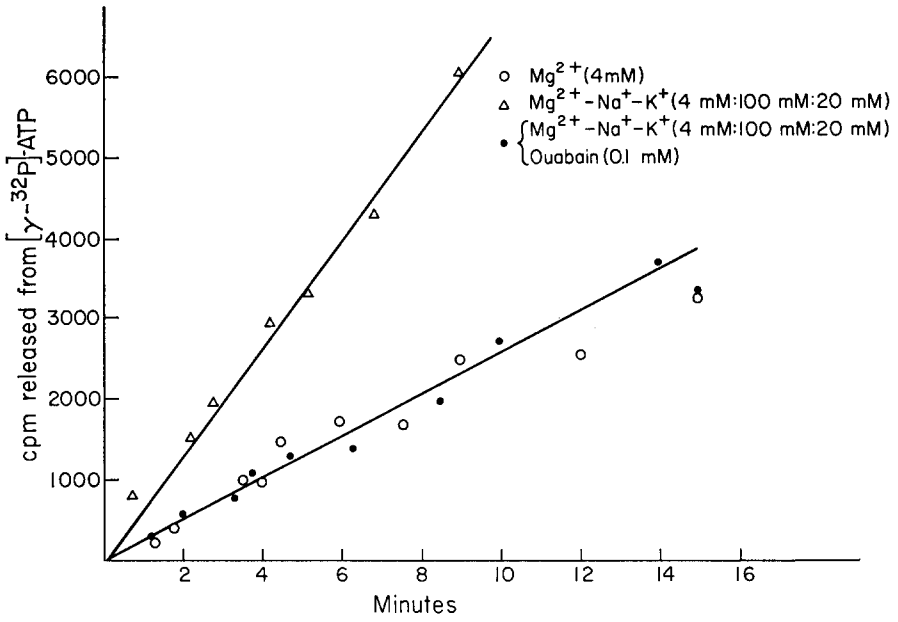



FIGURE 5 [$^{32}$P]-ATP hydrolysis by red cell ghosts. Effects of $Mg^{2+}$, $Mg^{2+}$–$Na^+$–$K^+$ and $Mg^{2+}$–$Na^+$–$K^+$ and ouabain. Incubation conditions: Tris–maleate (pH 7.0), 40 mM; $Mg^{2+}$:$Na^+$:$K^+$, 4:100:20 mM; [$^{32}$P]-ATP, 3 mM; ouabain, 0.1 mM; ghost protein, 2 mg/ml, temperature 37°. Aliquots were removed from the reaction mixture at appropriate times, treated with 10% $HClO_4$ and Norit and counted as described in the Methods section.

shows an activation–inhibition curve of ghost ATPase activity caused by increasing concentrations of SDS; a similar effect was seen with saponin, although the degree of activation was not so marked. There was no apparent change in the structure of the ghosts after preincubation in detergents at concentrations which produced maximal stimulation of ATPase activity, and all the ATPase activity remained sedimentable with the ghost membranes.

Incubation of ghosts in media containing cetyl pyridinium chloride resulted in varying degrees of inhibition of ATPase activity at all the concentrations tested.

TABLE I

*Substrate Specificity of Ghost ATPase*

| Nucleotide | μM $P_i$/mg protein/hr |
|---|---|
| ATP | 1.40 |
| CTP | 0.17 |
| GTP | 0.11 |
| UTP | 0.08 |
| ADP | 0 |

Red cell ghosts (1 mg protein/ml) were incubated for 30 min at 37° in Tris–maleate buffer (pH 7.0), 40 mM; $Mg^{2+}$:$Na^+$:$K^+$; 4:100:20 mM, and nucleoside triphosphates, each at 3 mM.

### *Effect of Metal Salts on ATPase Activity*

Since heavy metal salts are necessary trapping agents for the cytochemical procedures, experiments were carried out so that their effects on ghost ATPases could be determined. Four metals which give phosphates of low solubility were tested for their effects on total ATPase activity. Barium and nickel salts had no effect on the ghost ATPase activity at the concentrations tested, while lead and zinc showed approximately 50% inhibition of total ATPase in the 0.5 mM concentration range (Fig. 7). When lead was added to the incubation media in concentrations approaching (2.0 mM) those used in cytochemical procedures, only ~10% of the original ATPase activity was still demonstrable. This inhibition could be reversed in part by the addition of varying amounts of EDTA (Table II). $Pb^{2+}$ appeared to have a greater inhibitory effect on the Na–K–ATPase than on the Mg–ATPase (Fig. 8), yet the former activity was still demonstrable, and remained

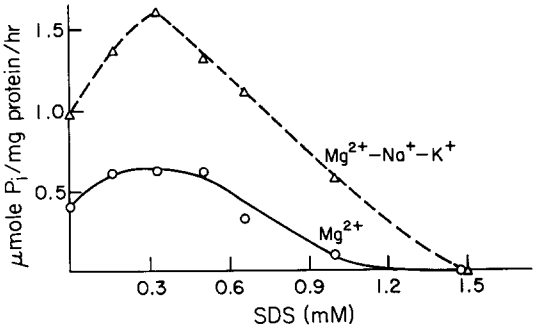



FIGURE 6 Effect of sodium dodecyl sulfate (SDS) on ghost ATPase in the presence of $Mg^{2+}$ and $Mg^{2+}$–$Na^+$–$K^+$. Ghosts (1 mg protein/ml) were incubated for 30 min at 37° in Tris–maleate (pH 7.0), 40 mM; $Mg^{2+}$:$Na^+$:$K^+$, 4:100:20 mM (or $Mg^{2+}$, 4 mM, alone); and ATP, 4 mM.

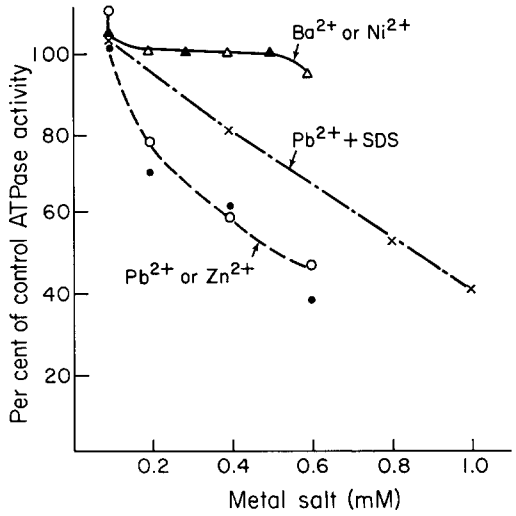



FIGURE 7 Effect of metal salts on ghosts ATPase. Ghosts (1 mg protein/ml) were incubated for 30 min at 37° in Tris–maleate buffer (pH 7.0), 40 mM; ATP, 3 mM, $Mg^{2+}$:$Na^+$:$K^+$, 4:100:20 mM; and metal salts at the concentrations indicated. $NiCl_2$, ▲——▲; $BaCl_2$, △——△; $ZnCl_2$, ○---○; and $Pb(NO_3)_2$, ●---●. The effect of 0.5 mM SDS on the inhibition of ghost ATPase by $Pb^{2+}$ is also indicated ($Pb^{2+}$ + SDS, x–-x).

fully sensitive to ouabain at $Pb^{2+}$ concentrations in the range of 0.1–0.8 mM.

### *Cytochemical Localization of ATPase Activity*

Cytochemical tests for ATPase activity were carried out by using the biochemical assay system described above supplemented with $Pb^{2+}$ in varying concentrations. In 0.1 mM $Pb(NO_3)_2$, the ghosts showed small precipitates associated with the inner surface of their membranes (Fig. 9). Some of these precipitates were located directly against the membrane while others appeared attached to the associated filamentous material. No precipitates were found on the outer surface of ghost membranes. Biochemically, both Mg–ATPase and $Na^+$–$K^+$–ATPase were fully active in this low $Pb^{2+}$ concentration (see Fig. 8).

Precipitates of reaction product were larger and more prominent on ghosts incubated in media containing 0.5 mM $Pb(NO_3)_2$, a concentration at which ~50% of the total ATPase was inhibited (Fig. 10). The distribution of precipitates remained the same, and the location appeared identical in preparations incubated in the presence of $Mg^{2+}$, $Mg^{2+}$–$Na^+$–$K^+$, or $Mg^{2+}$–$Na^+$–$K^+$–ouabain. In no case were significant amounts of precipitates attached to the outer surface of ghost membranes. The latter finding was somewhat surprising in the light of other studies (23–25). Since it was possible that an ATPase originally bound to the external surface of cell membranes was lost during the relatively long and elaborate preparation of the ghosts, intact red cells were lysed in a hypotonic ATPase medium containing 0.5 mM $Pb^{2+}$ and subsequently were incubated. In this case, as in the previous experiments, precipi-

TABLE II

*Reversal of $Pb^{2+}$ Inhibition of ATPase by EDTA*

| Incubation conditions | | CPM release from [$\gamma$ $^{32}$P]-ATP/mg protein/30 min |
|---|---|---|
| $Pb^{2+}$ | EDTA | |
| mM | mM | |
| Control ghost preparation | | 21,000 |
| 2.0 | | 2,800 |
| " | 0.2 | 3,200 |
| " | 0.6 | 4,400 |
| " | 1.0 | 8,400 |
| " | 2.0 | 15,400 |

Red cell ghosts (1 mg protein/ml) were incubated in Tris–maleate, 60 mM; $Mg^{2+}$:$Na^+$:$K^+$, 2:100:20, mM; ATP, 2 mM; and $Pb(NO_3)_2$, 2.0 mM at 4° for 5 min. Then varying amounts of EDTA, $Mg^{2+}$ (5 mM) and [$^{32}$P]–ATP (2 mM) were added and the mixtures were incubated at 37° for 30 min. Release of $^{32}$P from ATP was assayed as described in the Methods section.

tates were confined to the inner surface of the ghost membranes (Figs. 11 and 12).

### *Incubation with $Pb^{2+}$ and SDS*

SDS at certain concentrations was found to increase the total ATPase activity of the ghosts, as described earlier. In addition, SDS treatment resulted in less inhibition of ATPase activity by $Pb^{2+}$ (Fig. 7).

Electron micrographs of ghosts incubated in SDS–$Pb^{2+}$ showed the same localization of reaction product on the inner surface of the membranes (Figs. 13 and 14).

Some cytochemical experiments were also carried out with $^{32}P$-labeled ATP in order it could be determined whether the amount of reaction product bound to the ghost membranes in the presence of $Pb^{2+}$ and SDS was related to the total ATPase activity measured quantitatively. The amount of Pb–$^{32}$phosphate bound to the ghost membranes was measured by counting aliquots of washed ghosts after incubation with [$^{32}P$]-ATP in the presence of 0.4 mM $Pb^{2+}$ and 0.4 mM SDS. It was found (Table III) that Na–K–ATPase gave twice as much reaction product bound to the ghosts as did Mg–ATPase. Yet this increase was not immediately evident in the final micrographs of the cytochemical tests.

### *ATPase Activity of Glutaraldehyde-Fixed Ghosts*

Ghosts fixed in 0.5% glutaraldehyde before assay showed only 10% of the ATPase activity of unfixed preparations. In such fixed ghosts, the product of ATPase reaction appeared in the same location, i.e. the inner surface of the ghost membranes (Fig. 15), and was otherwise indistinguishable from that seen in unfixed ghost preparations. After fixation of ghosts in 2% glutaraldehyde, ATPase activity was barely detectable (less than 1–2% of control preparations). In the cytochemical tests, such ghosts showed only a few precipitates scattered throughout their interior.

### *Cytochemical Controls*

So that it could be determined whether nonspecific Pb precipitates form under the conditions used in our procedure, ghosts were incubated with ATP and $Pb^{2+}$ without any activating cations, or with ADP in a complete Na–K–ATPase medium, i.e. under conditions in which no enzymatic breakdown of ATP or related substrates could be demonstrated by quantitative biochemical assay. Precipitates were not found on the membranes in either case.

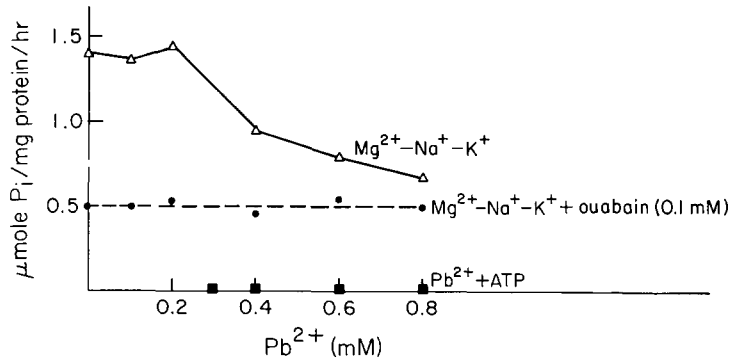



FIGURE 8 Selective inhibition of ghost Na–K–ATPase by $Pb^{2+}$. Incubation conditions as described for Fig. 6. $Pb^{2+}$ alone did not increase the nonenzymatic breakdown of ATP at the concentrations tested. Incubation of ghosts with $Mg^{2+}$ as the sole activating cation in the presence of $Pb^{2+}$ gave results identical with those shown in reactions containing $Mg^{2+}$–$Na^+$–$K^+$ and ouabain.

As noted earlier, no precipitates were found attached to the outer surface of the ghost membranes in any of the conditions investigated. This could be interpreted as indicating the absence of ATPase activity on the external surface of the red cell membrane, but it could be argued that the lack of precipitates resulted from their having been removed from the outside surfaces during processing for electron microscopy. So that this possibility could be investigated, Pb–phosphate precipitates were prepared and added to a suspension of ghosts which were subsequently processed for

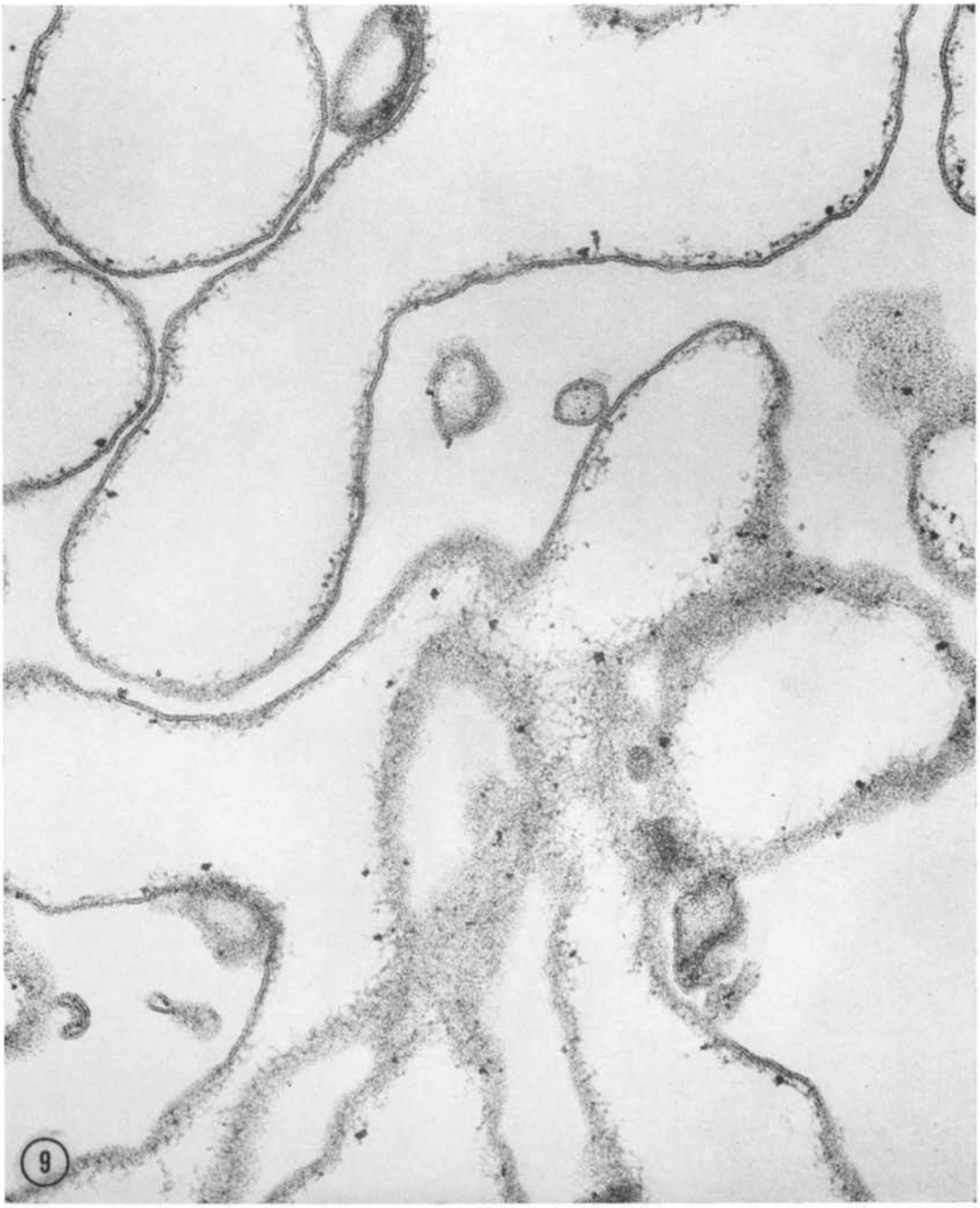
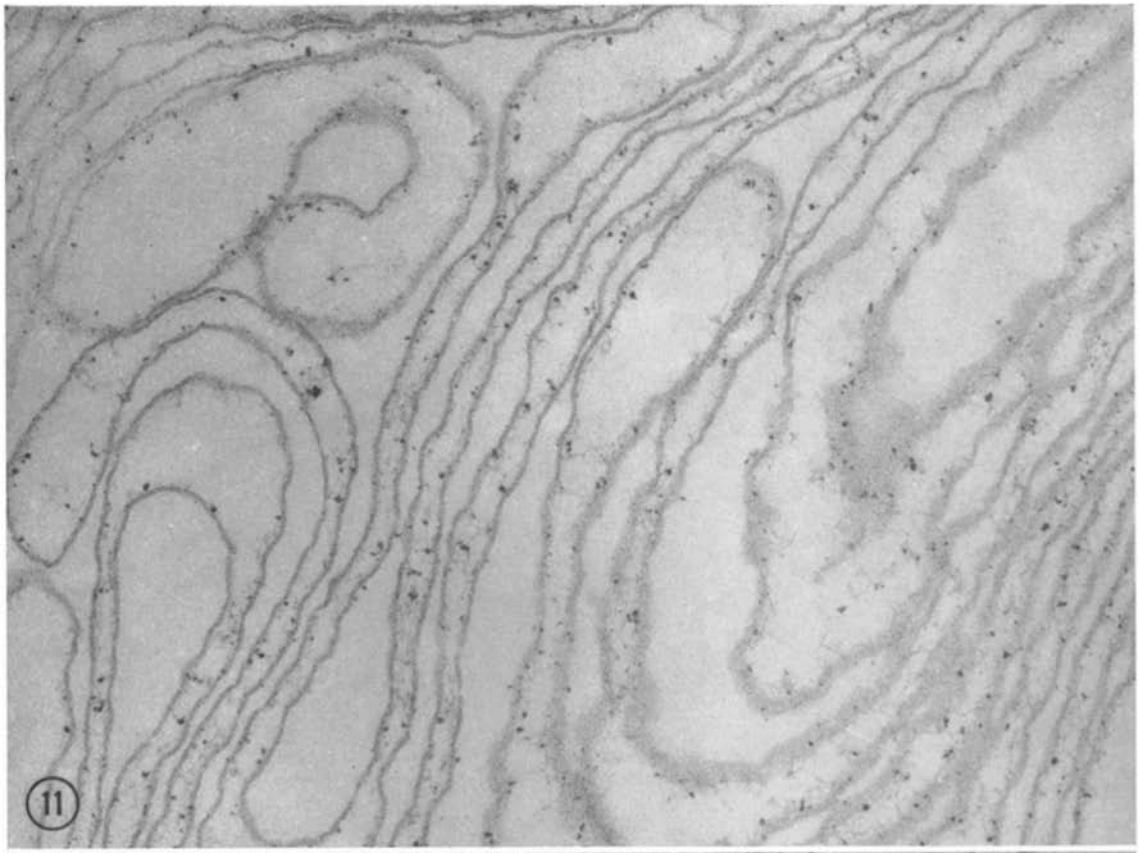
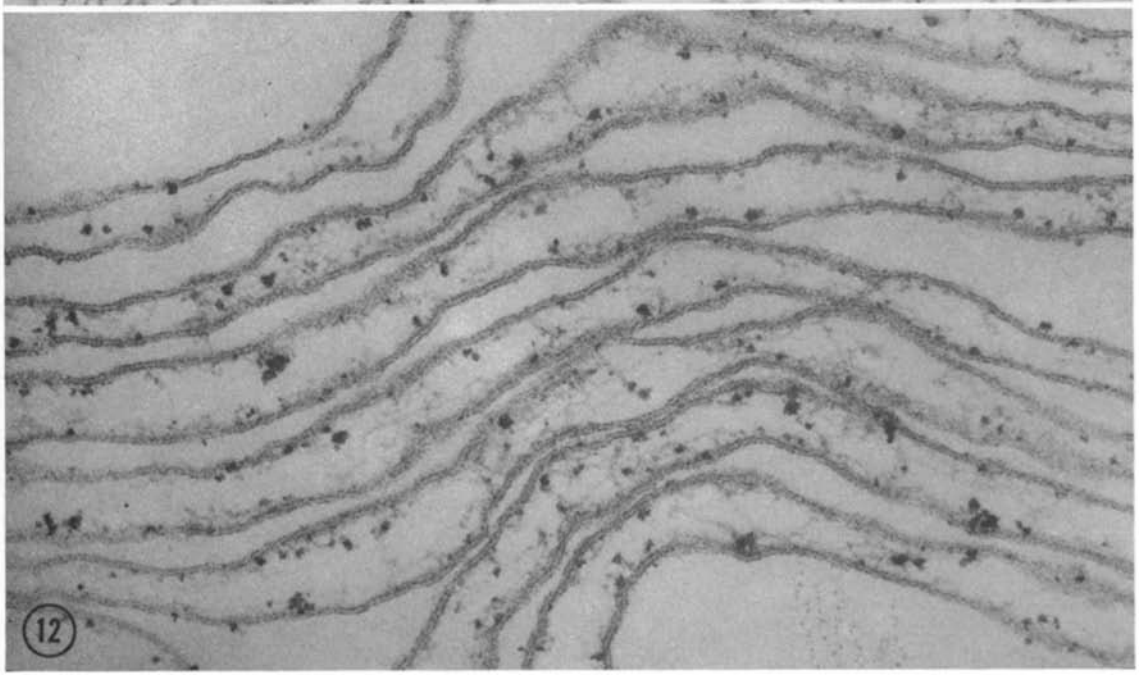


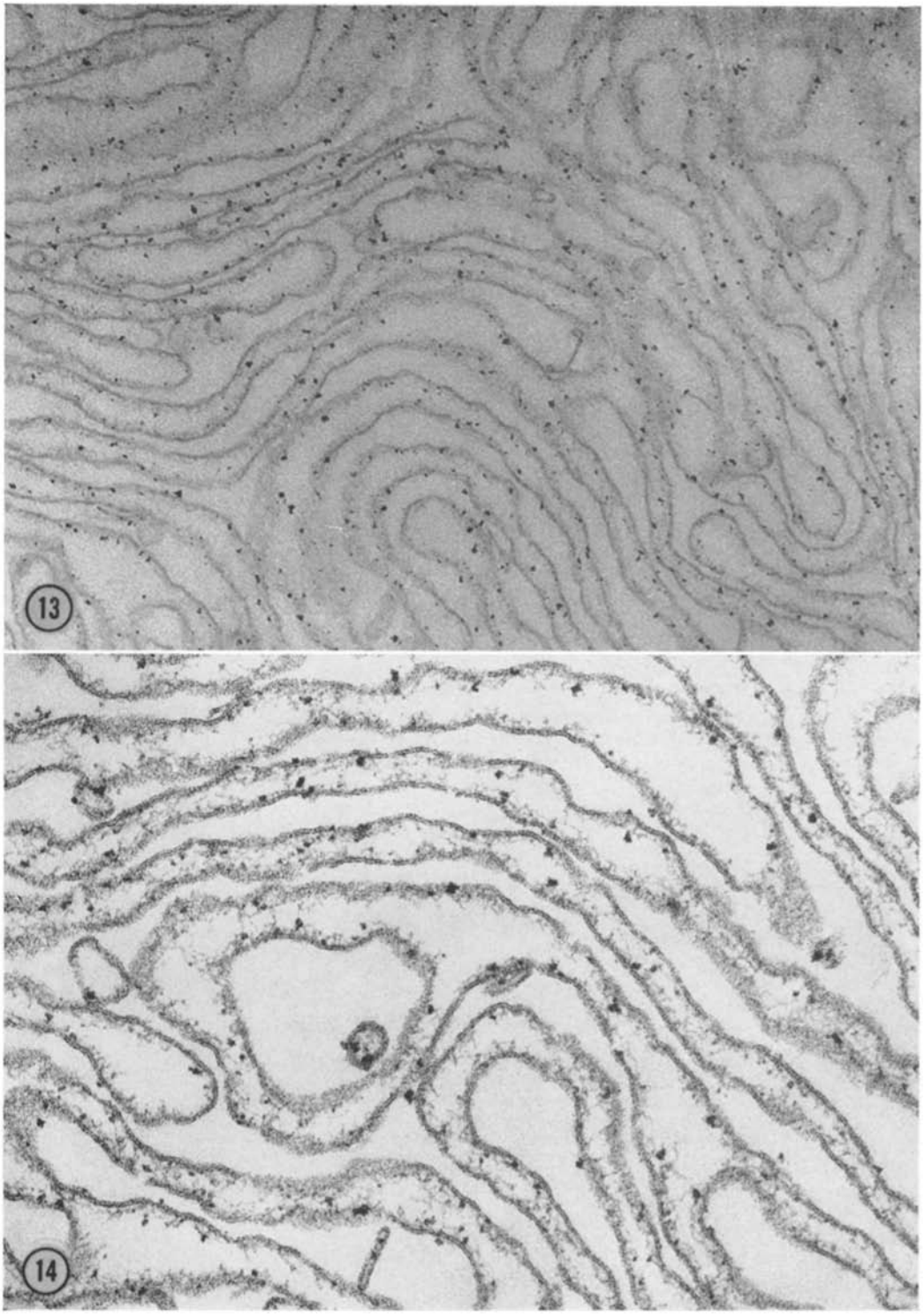

FIGURE 9 Red cell ghosts were incubated in a medium containing Tris–maleate, 40 mM; $Mg^{2+}$:$Na^{+}$:$K^{+}$, 4:100:20 mM; ATP, 4 mM, ouabain, 0.1 mM and Pb $(NO_3)_2$ 0.1 mM at 30° for 30 min and processed as described under Methods. Small precipitates are scattered along the inner surfaces of the ghosts and in association with filamentous material. × 90,000.


FIGURE 10 Red cell ghosts were incubated in a medium similar to that described for Fig. 9, but in this experiment, the $Pb^{2+}$ concentration was increased to 0.5 mM and the reaction was run at 37° for 30 min. Many precipitates of Pb–phosphate are seen along the inner surfaces of the ghost membranes. Only occasional precipitates are attached to the outside surfaces of the ghosts. × 60,000.




FIGURES 11 and 12 Intact red cells washed in 0.15 M NaCl were lysed in a hypotonic medium containing Tris–HCl buffer 2 mM (pH 7.2); ATP, 0.8 mM; $Mg^{2+}$, 0.8 mM; and $Pb(NO_3)_2$, 0.5 mM, and incubated at 37° for 15 min. Precipitates were localized only to the inner aspect of the ghost membrane and to the filamentous material attached to it. Fig. 11, × 60,000; Fig. 12, × 120,000.




FIGURES 13 and 14 Ghosts were incubated in the media described for Table III. Precipitates were localized to the inner surfaces of the ghost membranes regardless of whether the medium contained $Mg^{2+}$ or $Mg^{2+}$–$Na^{+}$–$K^{+}$. The appearances of the ghost membranes and the location of Pb-phosphate precipitates were identical with those found following incubation of ghosts in similar reaction media but without SDS. Fig. 13, × 60,000 (section unstained); Fig. 14, × 90,000.

electron microscopy in the same way as cytochemically reacted specimens. It was found that such exogenously added precipitates stuck to the ghosts throughout the preparation procedure and appeared attached to the outer surfaces (Fig. 16).

## DISCUSSION

### *General Approach*

In these experiments, we have tried to carry out cytochemical reactions under conditions similar to those currently used in biochemical assays for Mg–ATPase and Na–K–ATPase activities in various membrane preparations. This approach gave us the opportunity of determining systematically what type of activity, and how much of it, were available for cytochemical reaction in each experimental situation.

Most experiments were carried out on fresh, unfixed red cell ghosts, since we ascertained that the incubation of such ghosts did not result in detectable structure damage and since we found that fixation by glutaraldehyde caused marked ($\gtrsim$90%) ATPase inhibition.

The procedure we have used for the cytochemical demonstration of ATPase activity is a modification of the method proposed by Wachf stein and Meisel (12), and involves the addition o Pb ions to the incubation medium to trap the $P_i$ liberated upon ATP hydrolysis, as electron-opaque Pb–phosphate, at or near the site of its release.

### *ATPases Demonstrated*

Pb ions were found to inhibit the ATPase activity of the ghosts, especially their Na–K–ATPase, but the inhibition is concentration dependent and can be reduced to a tolerable level if the concentration of $Pb^{2+}$ in the incubation medium is brought down to 0.1–0.6 mM from the 2.0–3.6 mM currently used in cytochemical tests. In this range, biochemical assays indicate that Mg–ATPase is not affected and that a substantial fraction of Na–K–ATPase (~90% at 0.1 mM $Pb^{2+}$, and ~50% at 0.4 mM $Pb^{2+}$) is still available for the cytochemical reaction. Moreover, when ghosts were incubated in [$\gamma$-$^{32}$P]–ATP and $Pb^{2+}$, the amount of radioactivity bound to ghosts as reaction product was found to increase by ~100% upon $Na^+ + K^+$ stimulation (Na–K–ATPase) over that obtained in the presence of $Mg^{2+}$ only (Mg–ATPase). Yet, in the electron micrographs of these preparations, no great and consistent difference in the amount of Pb–phosphate precipitates was clearly seen. The increase is apparently too small to be easily detectable under unfavorable conditions caused by uncontrolled variations in the thickness of the sections and by some variation in the size and distribution of reaction product deposits.

TABLE III

*Effect of $Pb^{2+}$ on the Amount of $^{32}P$ Bound to Ghost Membranes Following Incubation with [$\gamma$-$^{32}P$]–ATP in the Presence of SDS*

| | Incubation conditions | | cmp $\gamma$-$^{32}$ P/mg protein/30min |
|---|---|---|---|
| Intact ghosts | $Mg^{2+}$ | $Pb^{2+}$ | 1,000 |
| Intact ghosts | $Mg^{2+} - Na^+ - K^+$ | $Pb^{2+}$ | 2,400 |
| Intact ghosts | No cations | $Pb^{2+}$ | 210 |
| Intact ghosts | $Mg^{2+} - Na^+ - K^+$ | No $Pb^{2+}$ | 530 |
| Heated ghosts (80°, 5 min) | $Mg^{2+} - Na^+ - K^+$ | $Pb^{2+}$ | 400 |

Red cell ghosts (1 mg protein/ml) were incubated in a medium containing Tris–maleate buffer (pH 7.0), 40 mM; either $Mg^{2+}$, 4 mM or $Mg^{2+}$:$Na^+$:$K^+$, 4:100:20 mM; [$^{32}$P]–ATP, 3 mM; SDS, 0.4 mM; and Pb $(NO_3)_2$, 0.4 mM (except when omitted). After incubation at 37° for 30 min, the preparations were chilled and centrifuged. The recovered ghosts were washed twice in Tris–maleate buffer, 50 mM. Membrane associated radioactivity was determined, as described in the Methods section.

### *ATPase Localization*

Regardless of the type of activity tested, i.e. Mg–ATPase, Na–K–ATPase, or Na–K–ATPase in the presence of ouabain, the deposits of reaction product were always found on the inner side of the ghost membrane, in immediate or close contact with the latter's inner dense leaflet. Deposits also appeared attached to, or in contact with, the fine filaments that form a feltwork on the inner aspect of the membrane, and in this location they oc-

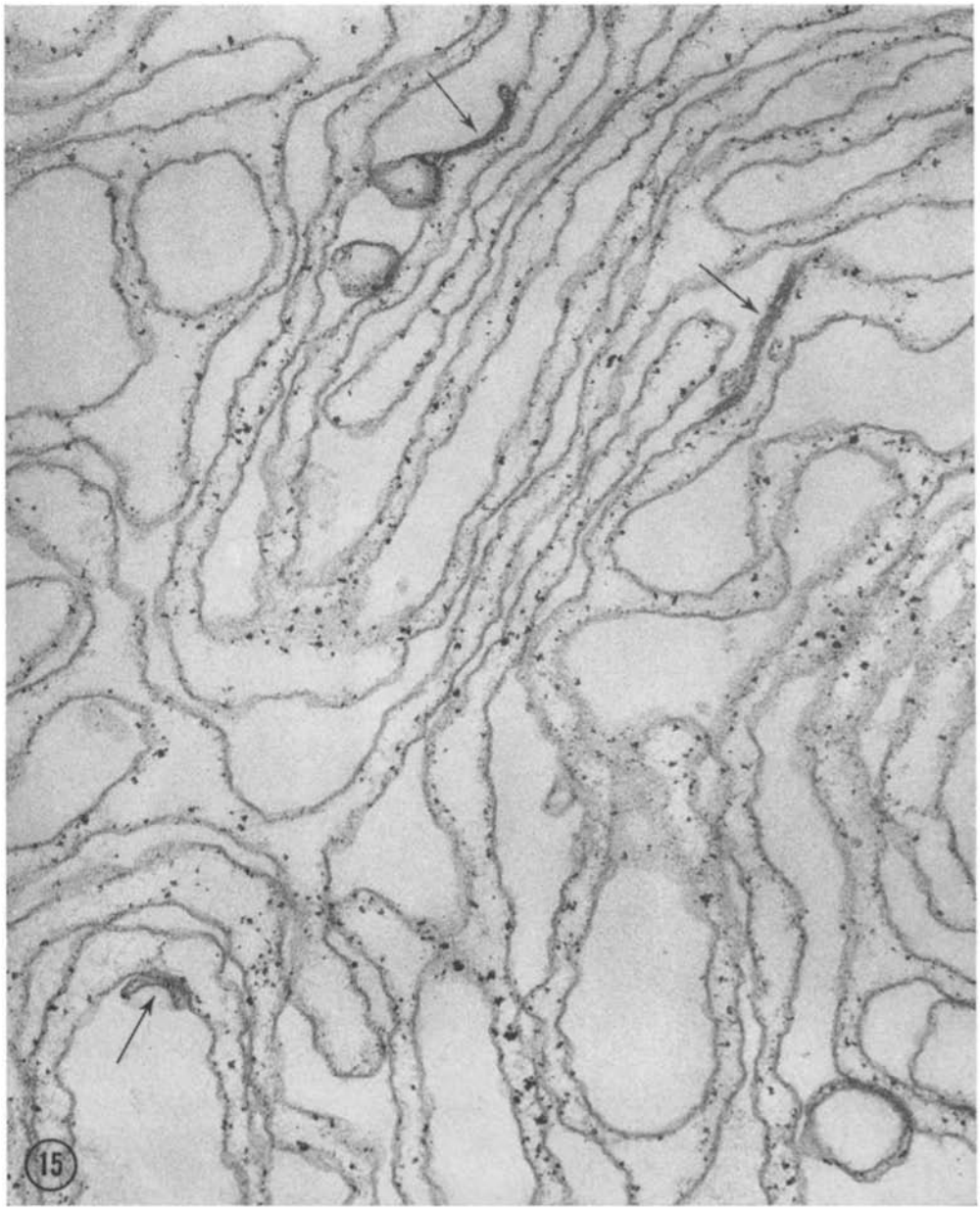



FIGURE 15 Red cell ghosts were fixed in 0.5% glutaraldehyde before incubation in a medium containing Tris–maleate, 40 mM (pH 7.0) $Mg^{2+}$:$Na^{+}$:$K^{+}$, 4:100:20 mM; and $Pb(NO_3)_2$, 0.5 mM at 37° for 30 min. Precipitates were confined to the inner surface of the ghosts and were identical with those seen in unfixed ghosts. Arrows mark myelin figures. × 60,000.

curred at distances up to ~300 A from the inner leaflet of the membrane. Since precipitates appeared at random over the inner surface of the ghost membrane, there was no indication that the ATPase activity is localized to a few selective sites. In addition the sites marked by reaction product deposits appear to be more numerous than estimated from physiological data (26).

Experiments carried out with red cells lysed in the incubation medium and with Pb–phosphate

added to the medium indicate that the absence of reaction products on the outer surface of the ghost membranes is not the result of losing either enzyme (during ghost preparation) or reaction product (during ghost processing for electron microscopy) from the outer leaflet of the membrane. Hence, it can be concluded that our procedure identifies the compartment in which $P_i$ is released upon enzymatic splitting of ATP and that this compartment is the same for ATPase activity under all

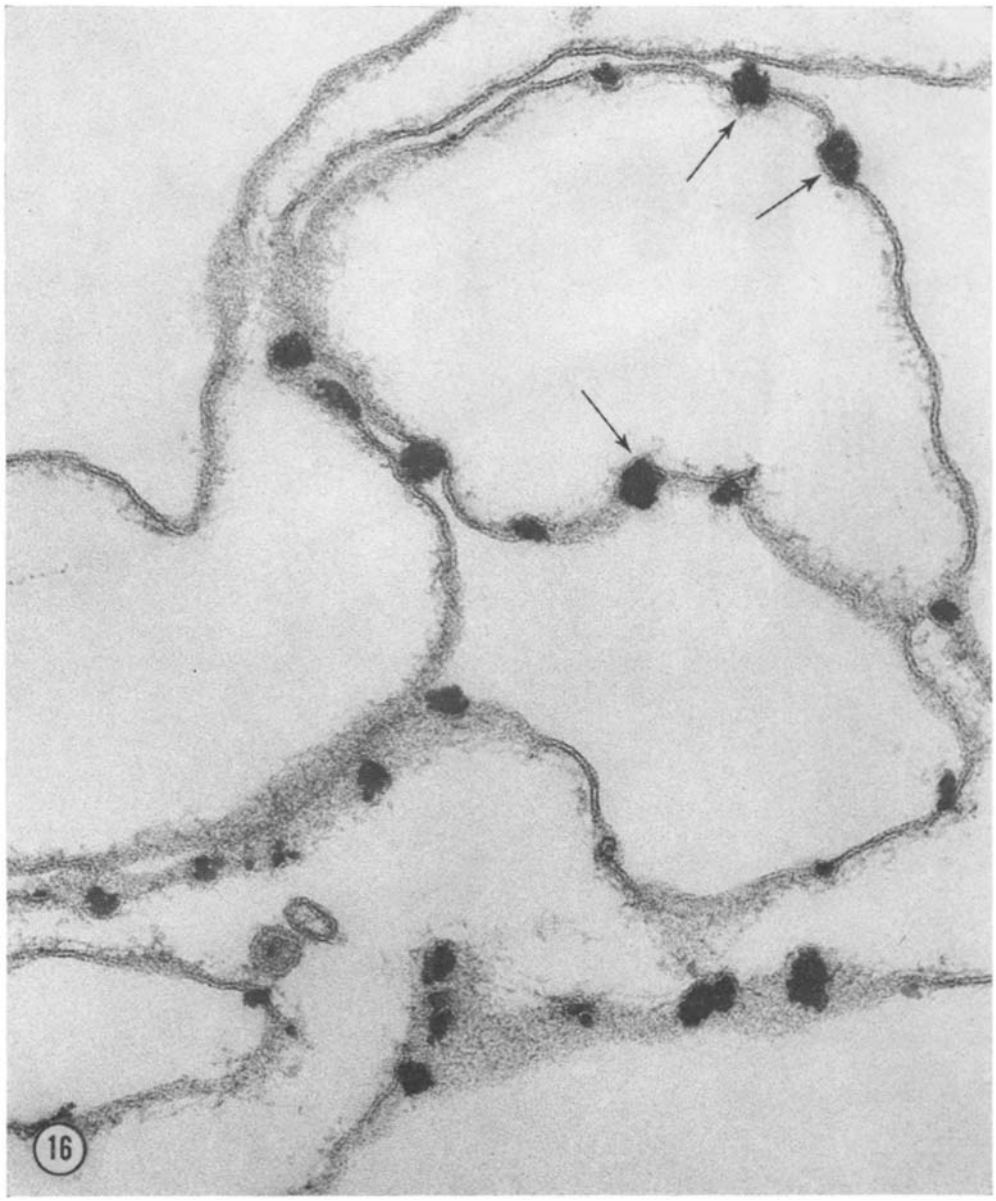



FIGURE 16 Red cell ghosts were incubated in a medium to which preformed Pb-phosphate precipitates were added in order that it could be determined whether such precipitates would stick to the ghost surfaces and remain adherent throughout processing for electron microscopy. Precipitates can be seen bound to the outside surface of the ghosts. The membrane appears to dimple in at the sites of precipitate adsorption (arrows). Such dimples were not seen in ghost incubated in ATPase media. × 120,000.

conditions tested. The enzyme appears to be part of the cell membrane and our evidence suggests that the enzyme site active in ATP hydrolysis is located in the inner dense leaflet of the plasmalemma.

The presence of reaction product deposits on the subjacent filamentous feltwork is more difficult to interpret: it may be caused by inefficient trapping of $P_i$, or it may indicate the existence of ATPase in the filaments. Preliminary observations on this filamentous material have been reported elsewhere (27) and studies thereon are continued.

### *Bearing on Previous Physiological Data*

To the extent to which they can be ascribed to Na–K–ATPase, our results are in agreement with physiological data which indicate that only intracellular ATP is used in cation transport. They also support transport mechanisms which postulate the intracellular release of the terminal $PO_4$ of ATP, regardless of other features involved such as a multistep reaction or a two step reaction in which a $Mg^{2+}$-activated phosphorylation reaction is followed by a $K^+$-activated phosphatase step (28). There is no agreement, however, between our findings and the view that Mg–ATPase (considered in this interpretation as nonspecific apyrase) is located on the outer surface of the membrane (10).

Since the location of reaction product is similar for Mg–ATPase and Na–K–ATPase, our observations provide no further insight into the relationship of these two activities. Recent studies suggest that Mg–ATPase can be converted into Na–K–ATPase by a variety of treatments (2, 15, 22, 29, 30), and such findings have been taken to indicate that the two activities represent different functional states of a single enzyme. Yet, since neither ATPase has been isolated and characterized in terms of its molecular properties, there is no firm evidence to establish whether one or two enzymes are present in these membranes.

### *Bearing on Previous Cytochemical Observations*

Previous attempts to study ATPase localization in mammalian red blood cells by lead-salt precipitation methods have produced results which do not agree with our findings. In these studies, precipitates were generally seen attached to the outside surfaces of red cells, and no reaction product was detected on the inside surface of the membrane. Since these observations were made on tissues fixed in aldehydes before cytochemical incubation, and since the latter was carried through in the standard Wachstein-Meisel medium which contains 3.6 mM $Pb(NO_3)_2$, it is likely that the absence of reaction product on the inner surface was the result of inhibition of ATPase activity by fixation, high $Pb^{2+}$ concentration, or both. As far as precipitates on the external surfaces of red cells are concerned, they were usually noticed on red cells *in situ*, i.e. within the blood vessels of different tissues. Since an ATPase associated with the cell membranes of the endothelium is not completely inhibited by aldehyde fixation (23–25), it is possible that reaction product generated by this ATPase was adsorbed nonspecifically to the red cells' outer surfaces. As described earlier, Pb–phosphate precipitates will adhere firmly to the outer surfaces of red cell ghosts and may react in the same way with the membranes of intact red cells. Alternatively, the reaction may be caused by phosphatases adsorbed from the plasma on the outer surfaces of the red cells. Yet, these considerations do not exclude the possibility that red cells of some species have ATPase activity on their external surface: in a recent study by Tooze (31), reaction products were localized to the outer surface of isolated amphibian red cells, while the outer surface of rat and rabbit red cells were nonreactive.

By lowering $Pb^{2+}$ concentration we have probably reduced the efficiency with which released $P_i$ is captured by $Pb^{2+}$. This could result in $P_i$ diffusion away from the site of its production before its capture and precipitation. Fortunately, in our experiments $Pb^{2+}$ concentration in the media was apparently sufficient to precipitate the released $P_i$ reasonably close to the sites of its production on the ghost membrane. We base this assumption on the following points.

(*a*) The localization of the Pb–phosphate precipitates was roughly the same regardless of the concentration of $Pb^{2+}$ in the incubation medium (over the range 0.1–0.8 mM).

(*b*) The amount of Pb–$^{32}$phosphate bound to the ghost membranes as ATPase reaction product was found to be proportional to the total ATPase activity of the reaction.

(*c*) Reaction products were found only on one side of the ghost membrane. Extensive $P_i$ diffusion from the sites of its production should give, if present, more precipitates on the outer surface of

the membranes since the ghosts are known to be permeable to $P_i$ (32).

The extent of $P_i$ diffusion away from its site of production probably depends on the relative activity of the enzymes involved. The specific ATPase activity of our ghost preparation, although similar to corresponding figures available in the literature, is rather low: 1.4 $\mu$M P/mg protein/hr. It is likely that this low activity has been an important factor in our successful use of such low $Pb^{2+}$ concentrations; this means that the conditions used in this study might not be suitable for other experimental systems.

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