*INACTIVATION OF ADENOSINE TRIPHOSPHATASE AND DISRUPTION OF RED CELL MEMBRANES BY TRYPSIN: PROTECTIVE EFFECT OF ADENOSINE TRIPHOSPHATE**

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In the course of a study of ATPase[1] activity of red cell ghost membranes, we made an attempt to separate the corresponding enzyme(s) from other membrane components by tryptic digestion. Since such a treatment has been used to obtain an enzymatically active fragment, heavy meromyosin,[2] from myosin ATPase, we were surprised to find that red cell ghost ATPase was rapidly inactivated by trypsin. Moreover, we found that ghost membranes were broken up into small fragments coincidentally with the tryptic inactivation of their ATPase. Enzyme inactivation as well as structural breakdown could be prevented by preincubating the ghosts in media containing ATP and Mg^{2+}. Ghosts protected in this way showed previously undescribed filaments associated with the inner aspect of their membranes. A preliminary account of these observations has appeared in reference 3.

Methods.—Guinea pig red cell ghosts were prepared and assayed for ATPase activity as indicated elsewhere.[4] For electron microscopy, specimens were either fixed, embedded and sectioned,[4] or negatively stained with 2 per cent phosphotungstic acid neutralized to pH 7.0 with 0.1 *N* KOH.

Results.—Effect of trypsin on ghost ATPase activity: Ghosts incubated with trypsin (10 μg or 50 μg/ml) in the absence of ATP and Mg^{2+} lost approximately 80 per cent of their total Mg-Na-K-dependent ATPase activity; similar preparations incubated in media to which ATP and Mg^{2+} were added shortly before addition of trypsin lost only 10–15 per cent of their total ATPase activity (Fig. 1). Preincubation in media containing ATP-Mg^{2+} was varied from one to ten minutes (before addition of trypsin) with no change in results.

Effect of trypsin on the structure of ghosts: Red cell ghosts incubated in media containing ATP and trypsin, i.e., under conditions that resulted in approximately 15 per cent loss of ATPase activity, had an over-all appearance[5] which in terms of size and shape was similar to that of control preparation. However, ghosts incubated in media containing trypsin without ATP, i.e., under conditions that caused a loss of approximately 80 per cent ATPase activity, fell apart into small fragments. The appearance of these two preparations is compared in the dark-field micrographs shown in Figures 2 and 3.

The protective effect of ATP on membrane structure was remarkably specific. Other nucleotides did not prevent gross disruption of ghost by trypsin (Table 1). ATP did not protect without Mg^{2+} (a condition under which ATP is not hydrolyzed by the ghost ATPase), and the reaction products of ATPase activity (ADP, inorganic phosphate) were not effective. None of the experimental conditions tested resulted in the disruption of ghosts without trypsin.

Electron microscopy of trypsinized ghosts: When studied by negative staining, ghosts trypsinized in the presence of ATP and Mg^{2+} showed bundles of filaments

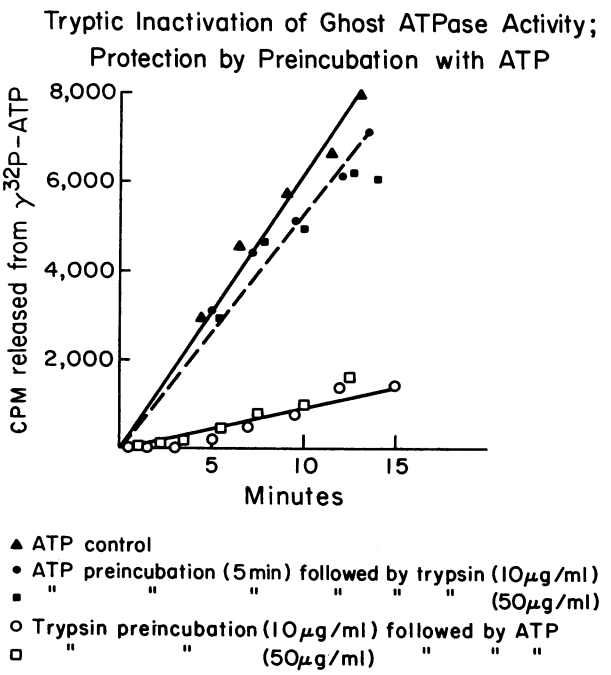


FIG. 1.—Red cell ghosts (1 mg protein) were incubated with Tris-HCl (40 mM), pH 7.0; $MgCl_2$, NaCl, KCl (4:100:20 mM); and ATP^{32} (4 mM) at 37° with trypsin added as indicated. ATP hydrolysis was measured by counting norit-treated aliquots of acidified reaction mixture.

extending across peripheral areas of their membranes (Fig. 4). The bundles seemed to be preferentially located on the inner side of the membrane. Individual filaments measured 40–50 Å in diameter and appeared helically coiled. Similar filaments were seen in ghosts incubated with trypsin and Mg^{2+} without ATP; in this case, they were considerably shorter in length and did not form broad, organized bands by lateral aggregation. No filaments were seen in ghosts incubated without trypsin.

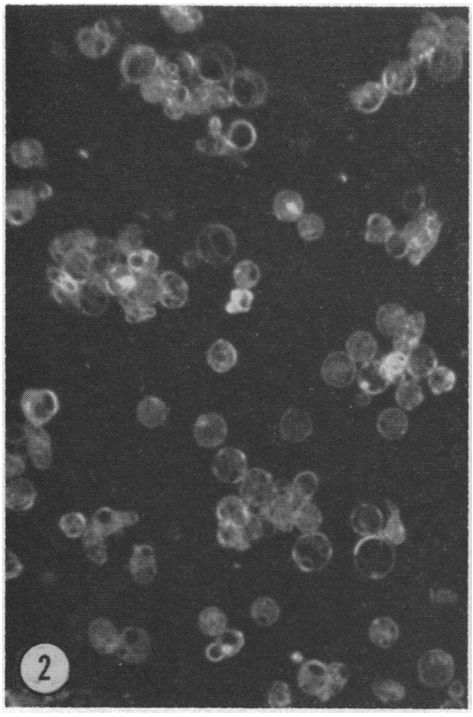
Extraction and in vitro polymerization of filaments from ghost membranes: Since the filaments found in trypsinized ghosts resembled the actin filaments isolated from

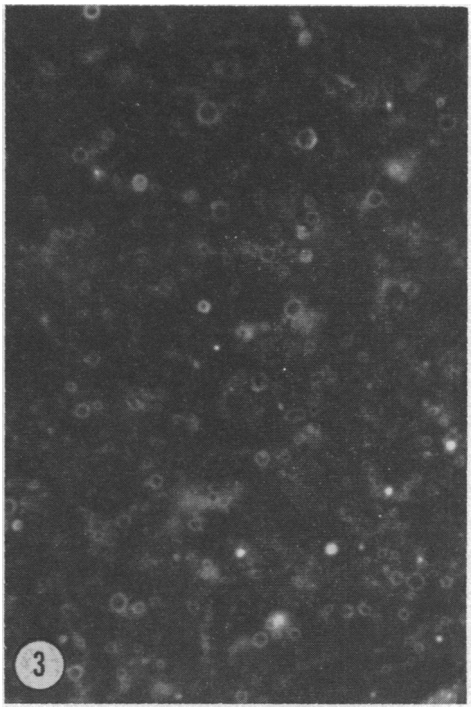
TABLE 1

PROTECTION OF GHOSTS AGAINST FRAGMENTATION BY TRYPSIN

	Incubation conditions	Appearance in Phase Microscopy: Intact	Fragmented
Trypsin	Mg + ATP	x	
"	Mg-Na-K + ATP	x	
"	" " + Ouabain	x	
"	Mg-Na-K		x
"	(No cations) ATP		x
"	Mg-Na-K + KH_2PO_4		x
"	" + ADP		x
"	" + GTP		x
"	" + CTP		x
"	" + UTP		x
0	All of the above	x	

For assay system see legend to Fig. 1. All nucleoside phosphates were used at a concentration of 4 mM.





FIGS. 2 (*left*) and 3 (*right*).—Dark field photomicrographs of red cell ghosts after incubation for 10 min at 37° with trypsin (10 μg/ml) in the presence of ATP and Mg^{2+} (Fig. 2) and after incubation with trypsin and Mg^{2+} without added ATP (Fig. 3). Both ~1000×.

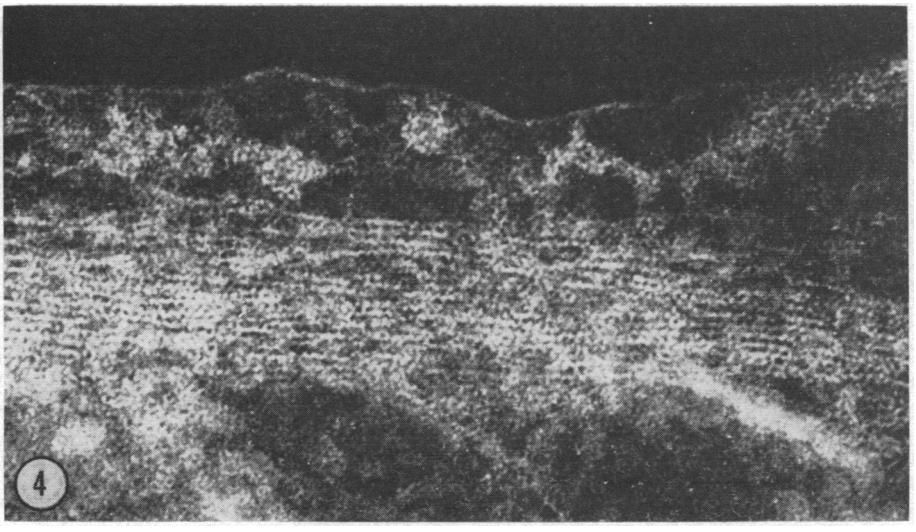


FIG. 4.—Electron micrograph of part of a red cell ghost after incubation for 15 min at 37° with trypsin (10 μg/ml) in the presence of ATP and Mg^{2+}. Coiled filaments are seen extending across part of the flattened membrane. Negatively stained with 2% K phosphotungstate. 240,000×.

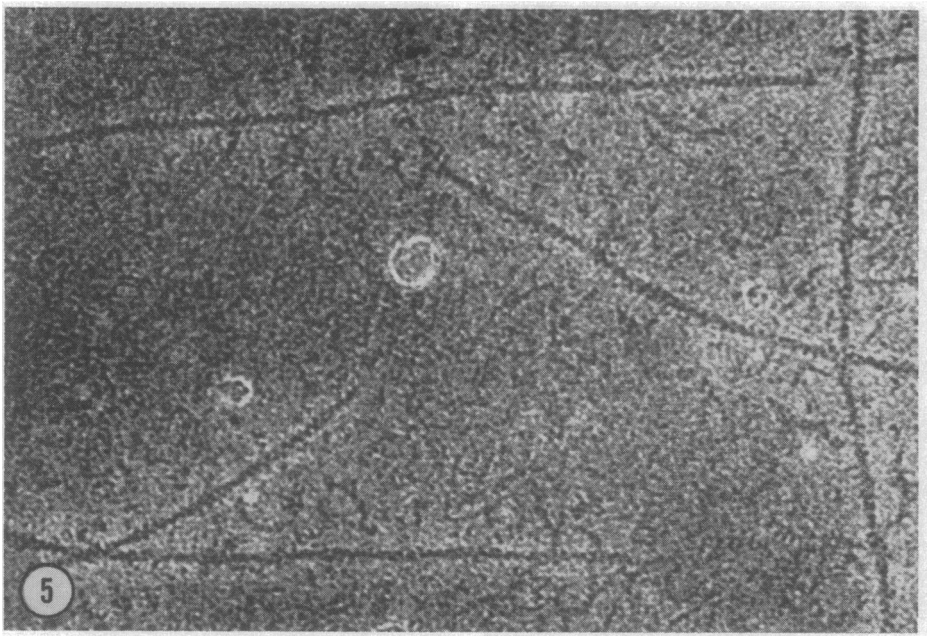


FIG. 5.—Electron micrograph of coiled filaments produced by incubating an ATP extract of trypsinized ghost membranes with Mg^{2+} and Tris-HCl at pH 7.0. Negatively stained with 2% K phosphotungstate (positively printed). 240,000×.

muscle,[6] we attempted to extract the ghost membranes by methods similar to those used for the extraction of muscle actin.

The extraction of trypsinized ghosts by low ionic strength media containing ATP solubilized part of the membrane proteins which were recovered in the supernate after centrifuging down the digested residues. Upon incubation with Mg^{2+} and ATP at neutral pH, this "solubilized" protein formed helically coiled filaments similar to those found in trypsinized ghosts (Fig. 5).

Discussion.—Our findings indicate that the incubation of red cell ghosts with trypsin results in concomitant ATPase inactivation and membrane fragmentation. Both effects are prevented by pretreatment of the ghosts with ATP and Mg^{2+}.

The protective effect of a substrate (in this case ATP) against inactivation of its corresponding enzyme has been observed in many different enzyme systems.[7] In our case, however, pretreatment of red cell ghosts with ATP protects the gross structure of the cell membrane against tryptic disruption, in addition to preventing the inactivation of ATPase. The conditions and specificity of the protective effect are identical in both cases. It is conceivable that the two effects, upon enzyme and structure, respectively, are coincidental and unrelated; yet, the findings suggest the possibility that the membrane-bound ATPase plays a role in maintaining the structural integrity of the membrane, in addition to its postulated function in active transport.

Bundles of filaments were found associated with ghost membranes as a result of trypsin treatment in the presence of ATP and Mg^{2+}. Such filaments were not found in nontrypsinized ghost membranes. One possible explanation for this

finding is that trypsin digests the membrane partially and thereby allows the negative stain to penetrate its depth and outline a pre-existing fibrillar component of the membrane. Alternatively, the trypsin might release a globular molecule which subsequently polymerizes to form the long filaments seen by negative staining. Ghosts examined in sections generally show filamentous material associated with the inner aspect of their membranes.[4] This material is no longer visible after extracting the ghosts in ATP at low ionic strength.[4] Hence, it is conceivable that these "native" filaments are the source of the monomer which upon polymerization in the presence of ATP and Mg^{2+} forms the bundles of helical filaments here described. The "native" filaments seen in nontrypsinized ghosts are reminiscent of the terminal web seen in various degrees of development in a large number of cell types; they may represent the minimal development of this structure in red blood cells.

The helical filaments found in trypsinized ghost membranes resemble closely the fibrillar form of muscle actin,[6] and the conditions under which such filaments were demonstrated in the membrane include the presence of ATP and Mg^{2+} at concentrations which favor the polymerization of muscle actin. Hence, the protein of these filaments might be related to actin. It remains to be seen, however, how close their relationship is and what function this protein performs (support? contraction?) in relation to the cell membrane.

Summary.—Trypsin digestion causes ATPase inactivation and structural breakdown in ghost membranes prepared from guinea pig red blood cells. Both effects can be prevented by preincubation in ATP and Mg^{2+}. The protection is highly specific: other nucleoside di- and triphosphates are inactive. Upon trypsin digestion, bundles of helically coiled filaments, morphologically reminiscent of actin, appear on the inner aspect of the ghost membranes.

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[1] The abbreviations used in this paper are: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

[2] Mihalyi, E., and A. G. Szent Györgyi, *J. Biol. Chem.*, **201**, 211 (1953).

[3] Marchesi, V. T., and G. E. Palade, *J. Cell Biol.*, **31**, 72A (1966).

[4] Marchesi, V. T., and G. E. Palade, *J. Cell Biol.*, in press.

[5] When examined by electron microscopy, such ghosts showed a few small membrane fragments within their limiting membranes.

[6] Hanson, J., and J. Lowy, *J. Mol. Biol.*, **6**, 46 (1963).

[7] Dixon, M., and E. C. Webb, *Enzymes* (New York: Academic Press, 1964).