The Hydrolysis of ATP and Related Nucleotides by Ehrlich Ascites Carcinoma Cells*

DONALD F. HOELZL WALLACH AND DONNA ULLREY

(Department of Biological Chemistry, Harvard Medical School, and Protein Foundation, Boston, Mass.)

SUMMARY

The ATPase of Ehrlich ascites carcinoma cells has been examined. The evidence presented indicates the following: (a) ATP hydrolysis occurs at the cell surface, (b) the cell membrane goes into the microsomal fraction upon cell rupture and differential centrifugation, (c) ATP hydrolysis depends upon the nature and concentration of external activator ions, (d) hydrolysis is not limited to ATP; other nucleoside triphosphates are attacked with equal vigor, and ADP and AMP are also hydrolyzed, (e) ATP hydrolysis appears to proceed in stepwise fashion via ADP and AMP to adenine and inorganic phosphorus, (f) ATP, but not ADP or ITP hydrolysis, is stimulated by 2,4-dinitrophenol, but this effect is reversed by glucose.

The presence on the surface of a variety of cells of enzymes capable of hydrolyzing adenosine triphosphate has been reported by several workers. Rothstein et al. have presented evidence for the localization of an adenosine triphosphatase on the surface of yeast (20) and of intestinal epithelium (21). Libet (13) and Abood et al. (1) have shown that the adenosine triphosphatase of the giant axon of the squid lies in the axon sheath rather than in the axoplasm. Essner et al. (9) have presented evidence localizing the adenosine triphosphatase of liver cells in the plasma membrane of the microvilli of bile cannaliculi; Spater et al. (24), also using cytochemical methods, have demonstrated adenosine triphosphatase activity in the plasma membranes of proximal renal tubular cells of a variety of species; and Novikoff (15, 16) has shown the presence of ATPase on the surface of a variety of tumor cells.

The stromal adenosine triphosphatase of erythrocytes has been the subject of considerable study (6, 8, 11, 27, 32). It has recently been implicated in the cation transport mechanisms of these cells by Post et al. (18). A potent adenosine triphosphatase on the surface of human leukocytes has been reported by Lukanova et al. (14). The hydrolysis of externally added adenosine triphosphate by Ehrlich ascites carcinoma cells was first recorded by Acs et al. (9).

The above studies suggest the possibility that adenosine triphosphate-hydrolyzing enzymes may exist as an integral part of the lipoprotein matrix of cell membranes. In the course of examining this hypothesis for the case of the Ehrlich ascites carcinoma, we have studied in some detail the cleavage of adenosine triphosphate and related substances by these cells.

MATERIALS AND METHODS

The tumor used was the hyperdiploid Ehrlich ascites carcinoma carried by weekly transfers in Swiss white mice. Tumors 5–8 days old and free of erythrocytes were used. For isolation of the cells 10 cc. of 0.15 N sodium chloride was injected into the peritoneal cavity of the host, and the resulting cell suspension was withdrawn. The cells were washed twice with 10 volumes of 0.15 N sodium chloride and were suspended to a concentration of 10 per cent in the medium composed of: NaCl, 0.142 M; KCl, 0.005 M; tris (hydroxymethyl)aminomethane, 0.087 M; pH 7.40.

Orthophosphate in the form of a sodium phosphate buffer, pH 7.4, was added to the above in appropriate amounts when the effects of phosphate ion were to be studied. The osmolality of the medium was then adjusted by omitting the appropriate amount of sodium chloride.

After a stock cell suspension of about 10 per...
percent had been prepared, a precise packed cell volume was obtained by centrifuging an aliquot in a Wintroub hematocrit tube at 4500 X g for 45 minutes. Other aliquots of the stock suspension were diluted to give final cell concentrations of 1-3 mg. of cells per ml. of suspension. As determined previously (30) 1 cc. packed cells is equivalent to 10^8 cells and 10^6 mg. of dry weight. Cells were incubated at 37° C. with gentle shaking for periods of 5 minutes to 2 hours. All experiments were carried out under aerobic conditions. Substrate concentrations ranged between 0.05 and 5.0 mm. Nucleotides used were obtained from Pabst and Co. After completion of incubation the cells were chilled, removed by centrifugation, and the supernatant fluid was analyzed for inorganic phosphorus (10) and 11-minute hydrolyzable phosphorus (4, 7) after deproteinization with 10 per cent trichloroacetic acid.

Because of the very low cell concentration (0.1-0.3 per cent by volume), more reliable data could be obtained by deproteinizing the chilled cell suspension directly, without prior removal of cells. In the bulk of the reported experiments, other than those where both extra- and intracellular labile and inorganic phosphorus were measured, this technic was employed but the appropriate cell and substrate blanks were also included. Disappearance of ATP from cell supernatant fluids was also measured directly by the method of Strehler and Trotter (25). Determinations were carried out in triplicate and showed no more than ±2 per cent variation in any one batch of cells. Whereas the ATPase activity was highly reproducible in any one tumor, it varied by ±24 per cent from tumor to tumor. Because of this, in all experiments the rate of production of inorganic phosphorus from ATP (0.5 mM initial concentration) was also measured directly by the method of Wachstein and Meisel (928). Air-dried, unfixed smears were used, since fixation in cold calcium-formol (3) led to marked inactivation of the ATPase.

**RESULTS**

*Association of the ATPase with sedimentable components of the cell.*—In 24 experiments intact Ehrlich ascites carcinoma cells produced 90 ± 22 μmoles inorganic phosphorus/ml cells/hr from externally added ATP (0.5 mM, initial concentration). The ATPase activity of homogenates was 2.5 times that of intact cells under identical conditions. The distribution of ATPase activity in the subcellular fractions is shown in Table 1. Ninety-two per cent of the ATPase activity was associated with sedimentable subcellular fractions, most of it in the microsomes. In contrast, most of the ADPase activity was found in the microsomal and soluble fractions.

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>ATP Activity</th>
<th>ADP Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nuclei</td>
<td>11.2 ± 5.2</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>28.9 ± 7.8</td>
<td>5.3 ± 2.8</td>
</tr>
<tr>
<td>Microsomes</td>
<td>56.3 ± 2.8</td>
<td>19.8 ± 5.9</td>
</tr>
<tr>
<td>Supernatant</td>
<td>7.6 ± 2.7</td>
<td>25.6 ± 4.0</td>
</tr>
<tr>
<td>2</td>
<td>104 ± 8.3</td>
<td>51.1 ± 5.6</td>
</tr>
</tbody>
</table>

Enzyme activity expressed relative to the rate of release of P, from ATP or ADP by whole homogenates. Homogenates and fractions incubated at 37° C. for 1 hour in 0.35 M sucrose containing Mg± (0.5 mM) and CN⁻ (1.0 mM). Initial substrate concentration 0.5 mM. Data show means and S.D.'s of three separate experiments.

The microsomal ATPase is associated with the microsomal membranes, which can be readily separated from the ribosomes by differential centrifugation. Thus, if whole microsomes are centrifuged for 18 hours at 105,000 X g in a sucrose gradient ranging from 42 to 50 per cent, 82 per cent of the ATPase and 78 per cent of the ADPase are found in the lighter half of the gradient, which also contains 77 per cent of the phospholipide but only 17 per cent of the RNA. The membrane preparation thus obtained can be further separated by centrifugal means into at least three distinct fractions, all of them free of RNA.²

Experiments on the effects of RNase upon microsomal ATPase support the above observa-

---

² Donald F. H. Wallach, to be published.
tions. Thus incubation of a dilute microsomal suspension with 0-0.02 per cent RNase at pH 7.4 and 37°C for 1 hour led to a loss of only 16 per cent of ATPase activity.

When RNase-treated microsomes were centrifuged at 105,000 × g for 30 minutes no ATPase was found in the supernatant, and less than 50 per cent of the original activity could be recovered in the pellet. This loss of ATPase activity in RNase-treated microsomal pellets has been reported previously by the authors (81) and is probably due to the aggregation of microsomal vesicles following RNase treatment, which makes resuspension of the pellets very difficult.

The ATPase of Ehrlich ascites carcinoma cells is readily demonstrable histochemically by the lead-nucleotide method of Wachstein and Meisel (28). As reported for other ascites cells by Novikoff (17), the ATPase activity of Ehrlich ascites carcinoma cells is associated with the cell surface and is demonstrable after 15 minutes of incubation at 37°C. On the other hand, no ADPase activity could be demonstrated histochemically even after 120 minutes, and hydrolysis of AMP appeared to be restricted to the nuclei and could be detected only after 60 minutes.³

To test whether any ATPase leaked from the cells during incubation, as is the case with some enzymes (5), cell suspensions were gently shaken aerobically for 1 hour at 37°C, and the cells were then removed by centrifugation. No ATPase activity was found in the cell supernatant, and cell activity was undiminished. There was also no leakage of ADPase into the medium.

Experiments were also performed to check whether ATP addition to the cell suspension medium influenced the intracellular labile and inorganic phosphate levels, but no significant effect was found. Thus, the average levels of intracellular inorganic phosphorus and acid-labile phosphorus in the absence of ATP were 10.6 ± 0.4 and 6.4 ± 0.6 μmoles/cc of cells, respectively. In the presence of ATP in the suspension medium at a concentration of 1 mM, the levels were 11.3 ± 0.4 and 5.6 ± 0.3, respectively.

Types of phosphate esters attacked by Ehrlich ascites carcinoma cells and paths of hydrolysis.—As shown in Table 2, all the nucleoside triphosphates were hydrolyzed with relatively little preference. Inorganic pyrophosphate, ADP, and AMP were also vigorously attacked, but β-glycerophosphate was split at only a very feeble rate.

As might be expected from these data, both of the phosphate anyhride linkages and the phosphate ester group of ATP are attacked by the ATPase of Ehrlich ascites carcinoma cells. This is illustrated in Chart 1, which shows the linear disappearance of 11-minute hydrolyzable phosphorus and appearance of inorganic phosphorus with time, the latter being greater than the loss of labile phosphorus.

It seems probable that a single enzyme attacks both ATP and ITP, since the rate of hydrolysis

### TABLE 2

HYDROLYSIS OF ATP AND RELATED SUBSTANCES BY EHRlich ASCITES CARCINOMA CELLS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>78 ± 9 (5)</td>
</tr>
<tr>
<td>AMP</td>
<td>92 ± 10 (4)</td>
</tr>
<tr>
<td>CTP</td>
<td>85 (1)</td>
</tr>
<tr>
<td>CTP</td>
<td>100</td>
</tr>
<tr>
<td>ITP</td>
<td>85 ± 14 (4)</td>
</tr>
<tr>
<td>UTP</td>
<td>80 (1)</td>
</tr>
<tr>
<td>PO₄</td>
<td>74 ± 94 (3)</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>13 ± 5 (2)</td>
</tr>
</tbody>
</table>

* Hydrolysis rates expressed relative to the rate of release of Pᵢ from ATP at an initial concentration of 0.5 mM. All nucleotides present at initial concentration of 0.5 mM. Activating ion = Mg²⁺ (0.5 mM). Cells incubated in standard medium. Data show means and S.D.'s for the number of experiments given in parentheses.
of ATP (.5 mM) in the presence of ITP (.5 mM) was only 37 per cent of that found with ATP alone. In any case, it appears that the two phosphate anhydride linkages are broken sequentially, followed by hydrolysis of the resulting phosphomonoester. This contention is supported by the inhibition exerted by ADP and AMP on ATP hydrolysis and of AMP on ADP hydrolysis (Table 3).

When the disappearance of ATP was followed by the luciferase technic, the rate of hydrolysis of ATP (0.5 mM, initial concentration) in the presence of an equimolar concentration of ADP was 85 per cent of that found in the absence of ADP. At the same time, the rate of production of inorganic phosphorus was 82 per cent of that from ATP alone and only 51 per cent of that expected if the hydrolysis of ATP and ADP were independent. Interestingly enough, an identical pattern could be demonstrated with ITP and ADP. These data could be explained by assuming that ATP and ADP are attacked by a single enzyme; but this seems improbable in view of the differing activator ion needs for ATP and for ADP hydrolysis. It is more probable that the inhibition of ATP hydrolysis by ADP is due to product inhibition. In either case our data are not consistent with the possibility that ADP breaks down via an adenylatekinase pathway.

Similar interrelationships can be shown to exist between ATP and AMP as well as ADP and AMP (Table 3). Thus, the inorganic phosphorus released was only 59 per cent of the expected value when ATP and AMP were both present at a concentration of .5 mM and only 37 per cent in the case of ADP and AMP (.5 mM). Even greater inhibition was found at the higher magnesium concentration, which excludes the possibility that those effects were due to magnesium binding by the nucleotides. In contrast to the above interdependence of the hydrolysis of ATP, ADP, and AMP, no significant effect of GDP on AMP hydrolysis could be shown, nor was there any effect of inorganic phosphate (1–10 mM) upon ATP hydrolysis.

The effect of substrate and cell concentration upon the rate of hydrolysis of ATP, ADP, and AMP.—As shown in Chart 2 for two magnesium concentrations, the rates of hydrolysis of ATP, ADP, and AMP at low initial concentrations appear to follow first-order kinetics. The strong dependence of activity on Mg** concentration at high concentrations of ATP and ADP, and particularly the inhibition of activity at high ATP levels, is best attributed to the Mg**-complexing action of ATP and ADP (log K for Mg-ATP = 3.47, and for Mg-ADP = 3.00, where K are the association constants) (25). This does not occur in the case of AMP, probably because of the low affinity of AMP for Mg** (log K = 1.69).

The rate of hydrolysis of these substrates varied linearly with the amount of cellular material present between 0.05 and 1.25 mg cells/ml cell suspension.

Ion dependence.—Contrary to the report by Acs et al., we found that the surface ATPase of Ehrlich ascites carcinoma cells is markedly dependent upon the presence of externally added divalent cations (Table 4). The activity without divalent cations was minimal. At the ion concentrations illustrated here (5 X 10^-4M) maximum activation occurred with Ca**. Mg** was only slightly less effective, Mn** considerably less, and Co** least so. Optimum activation occurred at concentrations of 10^-4M for both calcium and magnesium. At this concentration also, Ca** was slightly more effective than Mg**. No competition between Ca** and Mg** was found; on the contrary, their effects were additive. Thus, the activity obtained in the presence of 5 X 10^-4M calcium plus 5 X 10^-4M Mg** was equivalent to the activity obtained in the presence of 10^-5M Mg**. As might be expected the ATPase was completely inhibited by EDTA (10^-4M) and by fluoride ion (10^-4M).
In contrast to the marked ion dependence of the ATPase, adenosine diphosphatase activity was much less sensitive to externally added cations (Table 4). Nevertheless, definite stimulation was obtained with Ca++, Mg++, and Mn++, but not Co++. Maximal rates were again obtained at concentrations of Ca++ and Mg++ of 10⁻⁵M, but these were only about 30 per cent greater than without added activator cations. However EDTA (10⁻⁵M) fully inhibited ADPase activity, and the ADPases of the microsomes and soluble supernatant were entirely inactive without added activator ions.

The effect of glucose and of metabolic inhibitors.—The presence of glucose (10⁻⁵M) slightly, but consistently, reduced ATP hydrolysis by Ehrlich ascites carcinoma cells (Table 5). On the other hand, dinitrophenol (10⁻⁴ to 5 × 10⁻⁴M) without glucose increased ATPase activity by 20–47 per cent. At lower concentrations of this inhibitor (10⁻⁵ to 10⁻⁶M) no effect was observed. Of particular interest is the fact that dinitrophenol gave an identical increment in production of inorganic phosphorus when the experiment was carried out without externally added activator ion.

CN⁻ (10⁻⁵M) produced a similar but less marked effect. The stimulation of ATPase by dinitrophenol and by cyanide was more than completely reversed by the addition of glucose at concentrations of about 10⁻¹M. Thus, whereas dinitrophenol in the absence of glucose produced a 19 per cent stimulation of ATPase activity, the rate of hydrolysis of ATP in the presence of 10⁻¹M dinitrophenol.
and 10^{-3} M glucose was identical to that in the presence of glucose alone. Unlike the effect of dinitrophenol on ATP hydrolysis, no effect of this agent on the hydrolysis of ADP or ITP could be demonstrated.

**DISCUSSION**

Clearly, Ehrlich ascites carcinoma cells can hydrolyze externally added ATP as well as a variety of other phosphate esters. Two important questions arise in this regard: The first is whether the ATPase of these cells is on or a part of the surface membrane; the second is what the function of this and its associated enzyme systems might be.

As far as the localization of these enzymes is concerned, there are really only two possibilities: First, hydrolysis occurs within the cell—i.e., the substrates diffuse into the cell, are cleaved, and the products of hydrolysis diffuse out again. This suggestion, which has already been discussed for yeast by Rothstein (90), appears highly improbable here also. Certainly, mammalian cells are not readily permeable to polyphosphates such as the nucleotides under discussion, and even inorganic phosphorus moves into Ehrlich ascites carcinoma cells not by diffusion but by an active process (19). In any event, the problem is even more complex, since the cleavage sites are not in the soluble proteins underlying the surface membrane but in the microsomes and mitochondria. Even if it is assumed that the nucleotides can enter the cell and are hydrolyzed there, the problem of the movement of inorganic phosphorus from the cell remains. The present data show that externally added ATP does not affect internal ATP or inorganic phosphorus levels, yet inorganic phosphorus moves out of cells only very slowly2 even when external levels are zero and internal levels are raised artificially by DNP (28).

The second possibility is that cleavage of ATP occurs on the cell surface. Apart from the above considerations, three factors support this contention. The first is the histochemical localization of ATPase on the cell surface of the Ehrlich ascites carcinoma as well as other cells. The second is the striking dependence of ATPase activity upon the nature and concentration of external activator ions. In this connection it is also of interest that ADPase activity in these cells, which lies to a large extent in the 105,000 X g supernatant and which is relatively independent upon external activator ion concentration, cannot be visualized histochemically.

A third factor, consistent with the localization of ATPase activity on the surface membrane of these cells, is the finding that most of the nonmitochondrial ATPase is in the microsomal membranes. There is growing evidence that the plasma membranes of a variety of cells form small fragments upon cell rupture which sediment with the microsomes after differential centrifugation. Thus, Novikoff has shown that, upon cell rupture, the surface membranes of both a solid (16) and an ascites hepatoma (17) formed small vesicles not unlike the vesicles of the endoplasmic reticulum. The transplantation antigenicity of microsomes from the Novikoff hepatoma (16) and the human tumor H.Ep. #3 (26) have also been ascribed to the presence of cell membrane fragments in the microsomes. Finally, Wallach and Eylar (29) have recently found that the negative surface charge of Ehrlich ascites carcinoma cells is due largely to sialic acid and that most of the sialic acid of these cells is found in microsomal membranes following cell rupture. These studies again suggest that the surface membrane either sediments with the microsomal membranes after pressure homogenization or that it is continuous with them in the intact cell.

The function of cell surface ATPases is as yet unclear. Certainly the studies of Dunham (8), Whittam (32), and Post et al. implicate erythrocyte ATPase in cation transport. To date, our studies have not shown any definite effect upon ATPase activity of intact Ehrlich ascites cells other than that produced by the action of dinitrophenol and glucose. It appears that, in common with mitochondria, the surface ATPase is stimulated by dinitrophenol. However, in the latter case the stimulation is fully reversed by glucose. These data suggest that the activity of the surface ATPase in some way reflects intracellular energy requirements. Particularly cogent to this is the fact that DNP had no effect upon ITP or ADP cleavage.

In any event it appears probable that cell surface processes such as active transport, ameboid motion, and pinocytosis utilize ATP as an energy source and hence may act as "ATPases." Possibly these mechanisms are not rigidly oriented toward the cell interior and can thus utilize external ATP. Unfortunately, there is no information whether the energy yielded by surface ATP hydrolysis is actually used to drive cellular endergonic processes. It is also unknown what function is served by ADP and AMP hydrolysis, but our data may indicate that the ADPase is not a membrane-bound enzyme but is a soluble protein which becomes trapped in the microsomal vesicles during homogenization.
REFERENCES


Downloaded from cancerres.aacrjournals.org on April 12, 2017. © 1962 American Association for Cancer Research.
The Hydrolysis of ATP and Related Nucleotides by Ehrlich Ascites Carcinoma Cells

Donald F. Hoelzl Wallach and Donna Ullrey


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/22/2/228

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.