

THE EFFECTS OF MAGNESIUM ON NUCLEOSIDE PHOSPHATASE ACTIVITY IN FROG SKIN

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ABSTRACT

Histochemical tests, employing the Wachstein-Meisel medium, indicate that nucleoside triphosphatase activity is found predominantly in two areas of the frog skin epidermis: (1) in mitochondria, where activity is enhanced by dinitrophenol, Mg^{2+} dependent, but inhibited by fixation; and (2) apparently associated with cell membranes of the middle and outer portions of the epidermis, where activity is inhibited by Mg^{2+} , unaffected by dinitrophenol, and only slightly reduced by fixation. Spectrophotometric analysis shows that Mg^{2+} in the medium does not increase spontaneous hydrolysis of ATP, thus obviating the possible explanation that changes in substrate concentrations in the medium lead to alterations in the "staining" distributions. It is postulated that perhaps the two enzymes differ in their requirements for substrate—one requiring the polyphosphate to be in complexed form with Mg^{2+} , the other uncomplexed. Concentrations of Mg^{2+} required to inhibit cell membrane nucleoside triphosphatase activity also inhibit the electrical potential difference and short-circuit current of the frog skin. Although these observations might be taken as presumptive evidence of the cell membrane enzyme as a component of the ion pump system, because of certain dissimilarities with respect to the biochemists' "transport ATPase" and for other reasons discussed in the paper, any definite conclusions in this regard are premature.

INTRODUCTION

Histochemical studies of nucleoside phosphatase by the Wachstein and Meisel modification of the Gomori lead phosphate method have been undertaken by various workers (most recently by Novikoff, 12) in hopes of finding some relationships with the biochemists' "transport ATPase" (see review by Skou, 16). Not surprisingly, some of the properties of ATPases found by the histochemists differ from those found by the biochemists. This difference may be partly explained by the fact that, in one case, the enzymes are being studied in situ whereas in the other case, in an isolated state. Furthermore, as pointed out by Skou, the "transport ATPase" probably differs, in different animals, with respect to such factors as ability to hydrolyze different nucleoside phosphates, pH optima, Na^+K^+ activation, or ouabain sensitivity.

Nevertheless, there seems little doubt that ATP is an integral component of an ion transport system (see review by Whittam, 22). This reason alone warrants further histochemical investigations on the locus of the catalytic hydrolysis of ATP in material commonly used for the study of ion transport. In this respect, the frog skin provides an excellent system to study.

The interesting work of Farquhar and Palade (6) on frog skin led us to reexamine the question of sites of ATP hydrolysis. Although these workers reported that fixed sections incubated in the Wachstein-Meisel medium showed lead deposits in the intercellular spaces in the outer portions of the epidermis, mainly in the stratum granulosum and s. spinosum, our preliminary findings with unfixed sections did not consistently show deposi-

tion at these loci. Even when intercellular "staining" was obtained in the same area, it was markedly less pronounced than that shown in their photographs. We hoped that, by a systematic study, using fixed and unfixed tissues and varying the procedures of the Wachstein-Meisel method, we could resolve this disparity in observations and perhaps relate these findings to ion transport and bioelectric data taken from frog skin.

MATERIALS AND METHODS

The biological materials examined were the abdominal skins taken from summer adult *Rana pipiens* and *Rana catesbeiana*. In the bioelectric studies, individuals of these species were also studied in the fall. When tissues were fixed, pieces obtained from pithed frog were placed at 0°C in either 4% formal-calcium or 2.5% glutaraldehyde in 0.1 M cacodylate buffer for periods of 1/2-2 hr. Alternatively, tissues were also fixed after being cut on the cryostat.

Fresh or fixed tissues were cut on a cryostat at 10 μ , mounted on slides, and then incubated for 20 min at

room temperature in Wachstein-Meisel medium (80 mM Tris-maleate buffer pH 7.2, 0.84 mM ATP, 3.4 mM $\text{Pb}(\text{NO}_3)_2$, and 10 mM MgCl_2). In other experiments, ATP was substituted with 0.84 mM ITP, GTP, ADP, or Na β -glycerophosphate. All media were prepared fresh, with careful attention paid to purity of reagents and with cautious addition of $\text{Pb}(\text{NO}_3)_2$. Also, media were prepared without MgCl_2 and with 5 mM Mg^{2+} salt or with other divalent cations (MnCl_2 or CoCl_2) at 10 mM. Media in which $\text{Na}^+ - \text{K}^+$ ratios were varied (100/30, 200/60, 50/15, 100/0) were also prepared. Inhibitors placed in the media were *p*-chloromercuribenzoate (PCMB) (1 mM), ouabain (5 mM and 0.1 mM), and 2-4 dinitrophenol (1 mM).

The effect of Mg^{2+} on the frog skin electrical potential and short-circuit current, a measure of the skin's ability to pump Na^+ , was also tested in an apparatus essentially similar to that of Ussing and Zerahn (21). Silver-silver chloride electrodes were used for applying an external emf and calomel electrodes for recording the potential. To aerated Ringer's solution (105 mM NaCl, 5 mM KCl, 10 mM NaHCO_3 , 1 mM CaCl_2) was added 3 or 10 mM MgCl_2 . The Mg

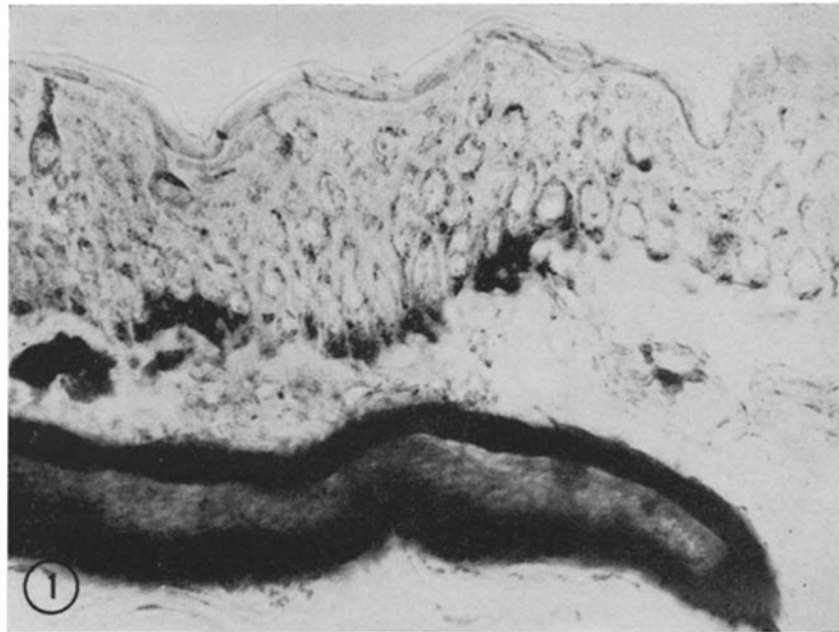


FIGURE 1 ATP medium with 1 mM dinitrophenol. Unfixed section of abdominal skin taken from *R. catesbeiana*. Reaction products are seen deposited in perinuclear mitochondria, predominantly in the s. germinativum layer. Slight staining is seen outlining cells in the outer layers of the epidermis. Dark areas at the junction of the epidermal and dermal layers are due to pigment granules. Darkly staining area at the bottom of the photograph shows nonenzymatic staining in the junction of the s. compactum and s. spongiosum. Mitochondria-rich cells, reported by Farquhar and Palade (6), seen in the upper left, show pronounced staining. $\times 540$.

Ringer's solution was usually added to both sides of the skin, but experiments were also carried out with Mg-Ringer's added to one side alone, with choline chloride added to the opposite side in order to balance the chloride ion concentrations.

The amount of spontaneously hydrolyzed ATP that might occur in the Wachstein-Meisel medium due to Mg^{2+} was analyzed by determining the amount of inorganic phosphate formed in a 1/2-hr period in media containing $Pb(NO_3)_2$ and $MgCl_2$, $Pb(NO_3)_2$ alone, and $MgCl_2$ alone. The method is a recent modification (10) of the Misson method for inorganic phosphate based upon the formation of a yellow phosphomolybdo-vanadate complex, and it allows for a determination of inorganic phosphate in the presence of ATP by spectrophotometric means. A Cary Model 14 recording spectrophotometer was utilized for this purpose, absorbency being read at 350 m μ . The Beer-Lambert Law was obeyed in our procedures in the range of concentrations employed in our standard curve of 1.4–43 $\mu g P_i/ml$.

RESULTS

When either fresh or fixed sections of frog skin are incubated in Wachstein-Meisel media either lacking substrate or containing glycerophosphate, lead deposits are seen primarily in two layers: the outer layer of the epidermis, the s. corneum, and at the junction of the s. spongiosum and s. compactum in the dermis. The latter layer has recently been reported by Taylor et al. (19) to contain heavy deposits of $Ca(PO_4)_2$ in the species of frog they investigated, *Rana catesbeiana*. Other workers (8) have also reported heavy staining in this junctional area when employing the Gomori method. Thus, the staining in this layer at least cannot be construed as enzymatic when sections are incubated in ATP media. The staining in the cornified layer has not been identified, but perhaps may represent deposits due to mucopolysaccharides contained in the mucous coating of the skin.

When Mg^{2+} is incorporated into media in which fresh sections are incubated, reaction products are seen primarily in what appear to be mitochondria, with greatest concentration in the s. germinativum layer of the epidermis. To a great deal less extent, faint intercellular deposits may be seen in the outer layers of epithelial cells. The reaction products in mitochondria may be seen more clearly in photographs (Fig. 1) when dinitrophenol is incorporated in the incubating medium. Dinitrophenol stimulates the ATPase activity of the mitochondria as it does in other tissues (7, 15). Mitochondrial ATPase activity is known to be

dependent on Mg^{2+} , and in its absence histochemical reactions show, as expected, little or no reaction product in the mitochondria (Figs. 2, 3). Also, as is well known, fixation destroys mitochondrial activity (Figs. 4, 5), regardless of the fixative employed.

When Mg^{2+} is omitted from the media, a striking effect is observed. While mitochondrial staining disappears, as indicated above, intercellular staining becomes dramatically evident whether the tissue is fresh (Figs. 2, 3) or fixed (Figs. 4, 5), and corresponds in its distribution more typically to the results obtained by Farquhar and Palade (6). Dinitrophenol does not stimulate this effect. That this staining is enzymatic is further borne out by observations on serial sections on which incubations were carried out with intermediate concentrations of substrate (ATP) or inhibitor (Mg^{2+}). In all cases, intermediate concentrations of substrate or inhibitor produced intermediate levels of intercellular deposits. If ADP is substituted for ATP in the media, little or no deposits are seen (Fig. 6). ITP (Fig. 7) or GTP will apparently substitute for ATP in the media.

PCMB completely inhibited mitochondrial staining. The intercellular nucleoside triphosphatase, however, was not visibly inhibited by either PCMB or ouabain in the two concentrations used. Also, variation of Na^+ or K^+ levels in the media did not show obvious results in distribution or degree of lead deposition. Mn^{2+} and Co^{2+} seemed to produce the same effect as Mg^{2+} , perhaps giving greater inhibition.

Table I shows that, when Ringer's solution containing 10 mM $MgCl_2$ (the concentration employed routinely in the Wachstein-Meisel media) is used to bathe both sides of an isolated frog skin in a lucite chamber device, the short-circuit current and electrical potential difference are reversibly depressed in the two species of frogs used in these studies. The effect when Mg^{2+} is added to the outside only is similar, but in the case of *R. catesbeiana* it is not so pronounced for reasons to be discussed below. In the species of frogs used in European studies, *R. temporaria*, divalent cations, including Mg^{2+} , when added to the outside have similar inhibitory effects (5). However, as observed by these workers and by us, seasonal variations are evident with this Mg^{2+} effect. Thus, for example, *R. catesbeiana* in late fall shows less sensitivity to Mg^{2+} inhibition, and the effect takes a longer period of time to become fully developed

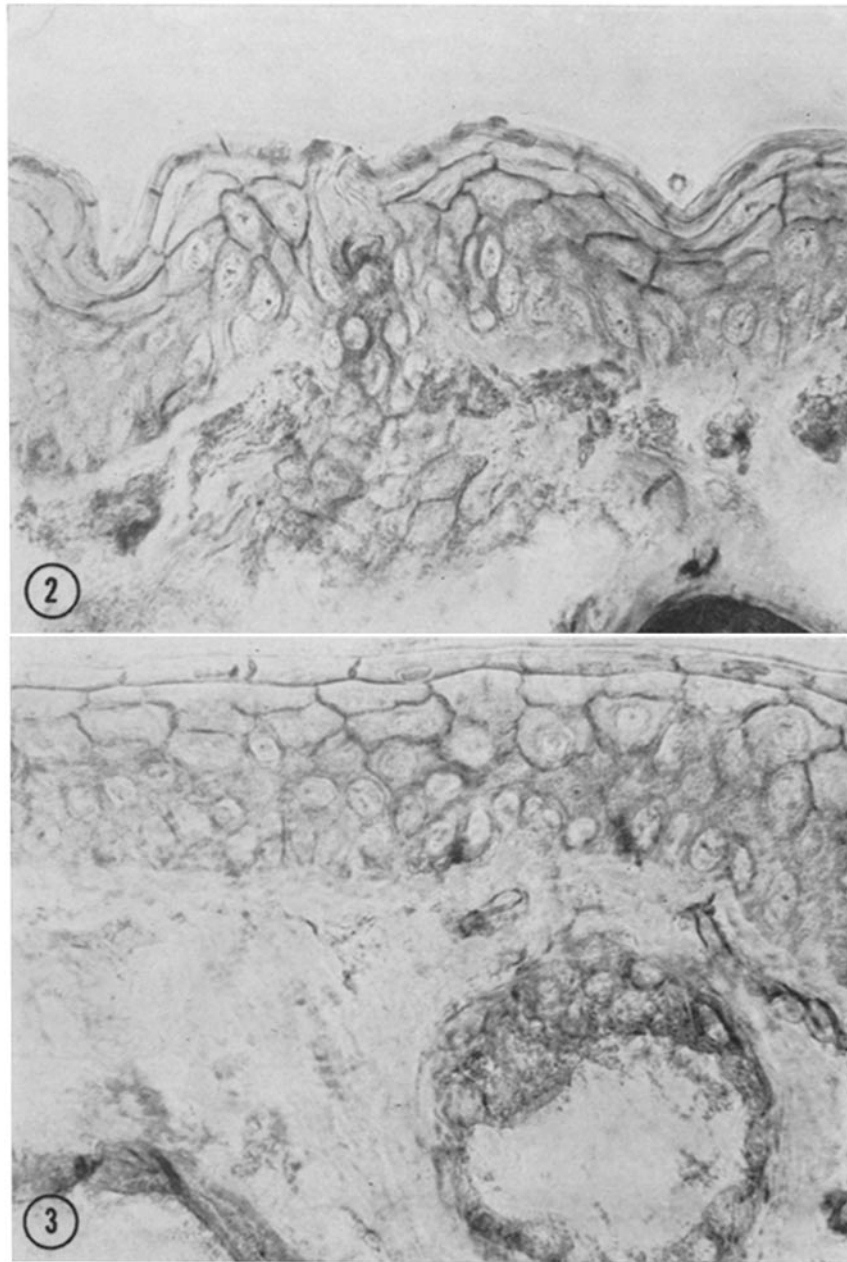


FIGURE 2 ATP medium lacking Mg^{2+} . Unfixed section taken from same skin. Marked staining is observed primarily outlining cells in middle and outer layers of the epidermis. Mitochondrial staining is not apparent. In the middle of the photograph is seen a more darkly staining, vertical area showing the body and neck portions of a mucous gland. $\times 540$.

FIGURE 3 Same as Fig. 2, but different area of the skin. Additional observation to be made is the pronounced staining appearing in the mucous gland and blood vessels to the right of the gland. The relationship, if any, between glandular activity and intercellular staining is unknown. Glandular staining, however, is due to nucleoside triphosphatase activity. $\times 540$.

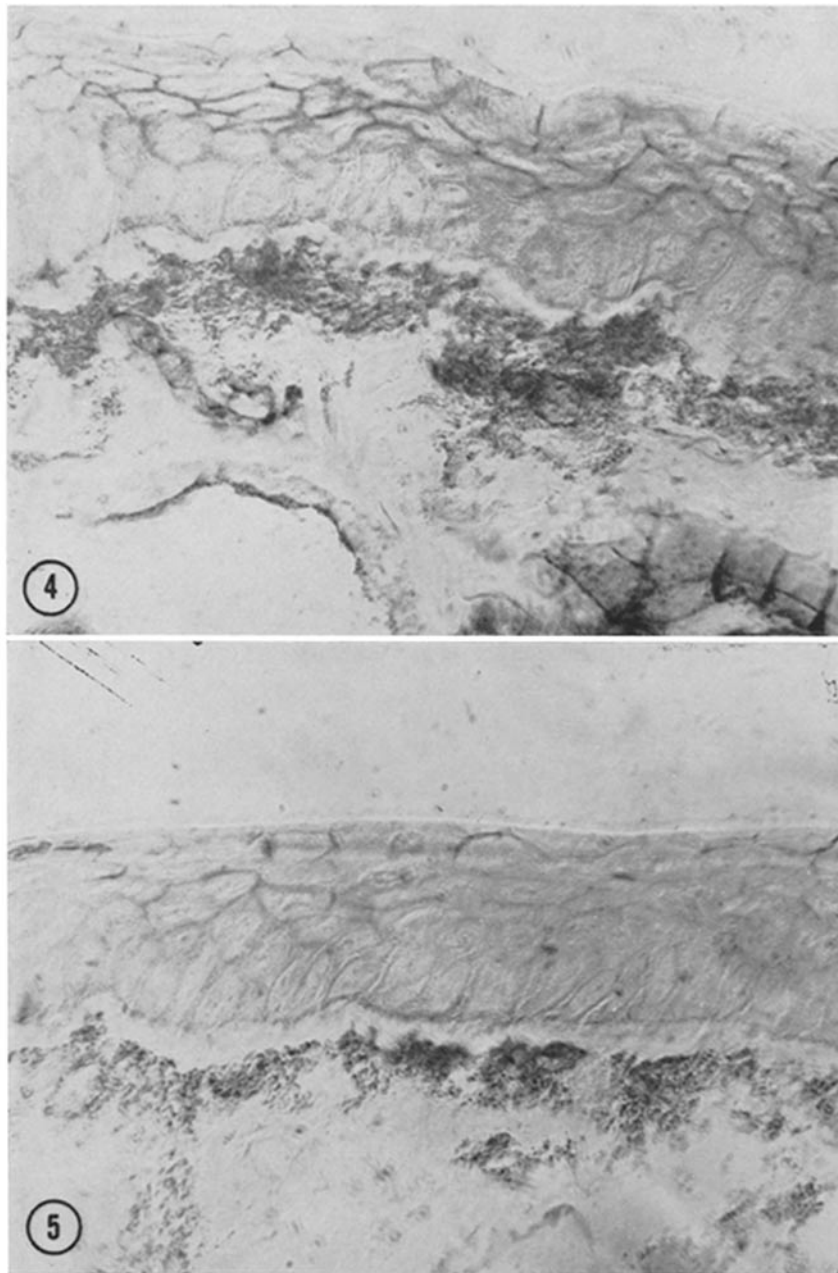


FIGURE 4 ATP medium without Mg^{2+} . Same skin, fixed in 4% formol-Ca. Intercellular staining appears in same areas as in Fig. 2, but is diminished in intensity. Due to fixation, mitochondrial staining is lacking. Glutaraldehyde fixation slightly decreases the staining intensities. Cavity of a gland is seen in lower left. $\times 540$.

FIGURE 5 ATP medium with Mg^{2+} . Same skin, fixed in 4% formol-Ca. Intercellular staining is much reduced. Mitochondrial staining appears to be lacking. Pigment granules are seen immediately beneath the epidermal layer. $\times 540$.

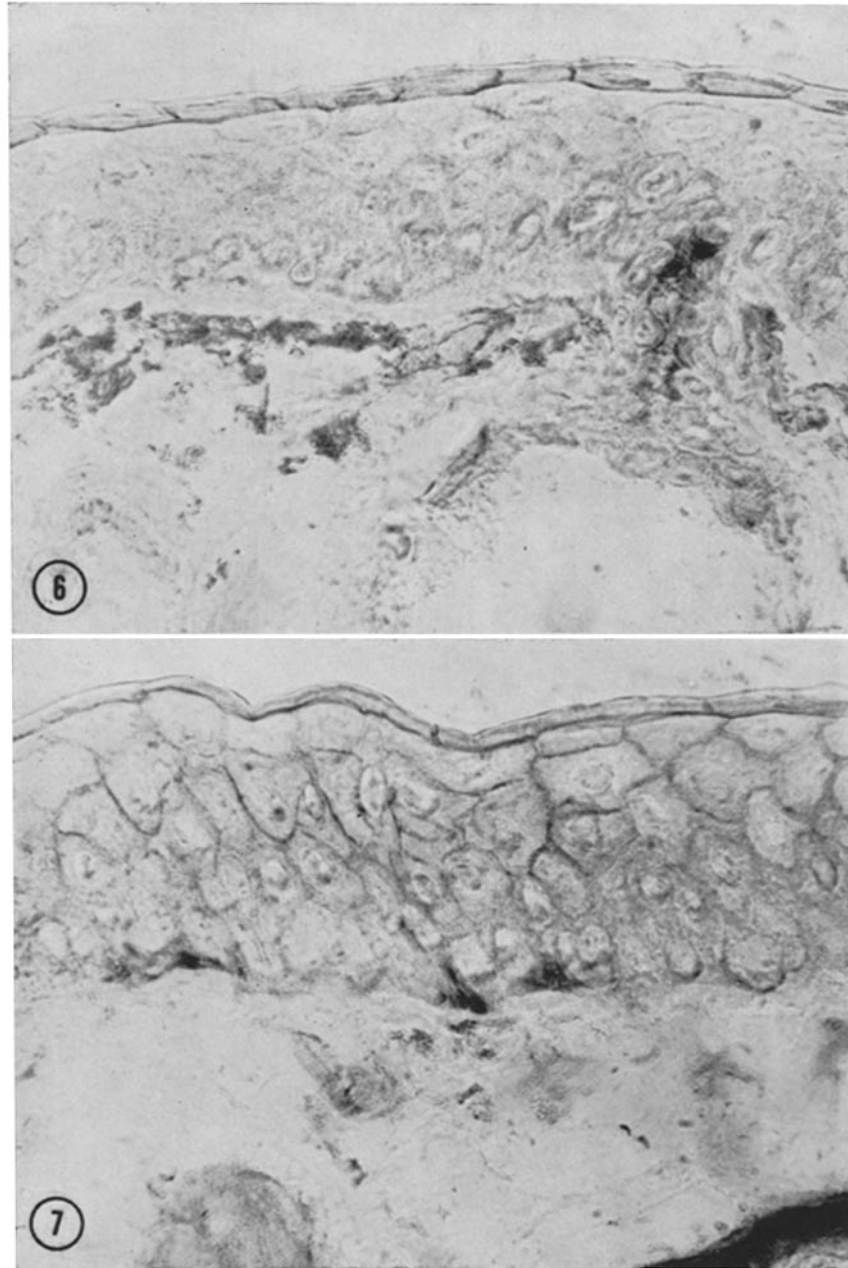


FIGURE 6 ADP medium without Mg. Same skin, unfixed. Only traces of intercellular staining remain. $\times 540$.

FIGURE 7 ITP medium without Mg. Same skin, unfixed. Intercellular staining appears slightly reduced (cf. Figs. 2 and 3). $\times 540$.

TABLE I
The Effect of Addition of 10 mM Mg²⁺ to Ringer's Bathing Solutions

Species	Season	No. animals	Side of application	Mean and range of % potential difference remaining after treatment	Mean and range of % short-circuit current remaining after treatment
<i>R. catesbeiana</i>	Summer	7	Both	51.5 (44.5—58.2)	53.0 (52.4—60.0)
<i>R. catesbeiana</i>	Fall	8	Outside	—	80.5 (63.0—106)
<i>R. pipiens</i>	Fall	16	Both	—	54.3 (41.6—68.4)
<i>R. pipiens</i>	Fall	18	Outside	64.8 (26.0—84.2)	52.2 (31.5—75.0)
<i>R. pipiens</i>	Fall	8	Inside	—	98.3 (83.3—121)

(30–60 min in comparison to 5–10). The seasonal and presumably hormonal variations that alter the Mg²⁺ inhibitory effect and the significance of Mg²⁺ effect when the ion is applied only to the inside require further investigation.

That the differences observed in enzyme localizations due to Mg²⁺ are not attributable to the lowering in substrate concentrations brought about by increased hydrolysis of ATP, was shown by the spectrophotometric analysis. The addition of Mg²⁺ did not increase the amount of hydrolysis of ATP beyond that caused by Pb²⁺. Within a 30-min period, the inorganic phosphate levels in the media represented about a 10% total possible hydrolysis of the terminal phosphates of the ATP. Our findings are in agreement with previous findings of Rogers¹ and show that Pb²⁺ alone increases the spontaneous hydrolysis of ATP in the medium. The Mg²⁺ effect on hydrolysis reported by other workers (11) apparently has no effect on the hydrolysis under the conditions of the Wachstein-Meisel procedure.

DISCUSSION

One of the most pronounced differences between the biochemists' ATPase and the histochemists' nucleoside triphosphatase is the former's strict requirements for Na⁺, K⁺, Mg²⁺, and ATP in critical, relative concentrations and for specific inhibition in the presence of ouabain. Histochemical studies differ from biochemical studies primarily in terms of incubating media used and method of tissue preparation. Because of the method of tissue preparation for histochemical studies, there is the distinct possibility that Na⁺-K⁺ activation and ouabain inhibition would not be demonstrable because of a greater possibility of the occurrence of

bound Na⁺ and K⁺. This has been shown to occur in some biochemical studies in which Na⁺-K⁺ activation and ouabain inhibition were masked by the presence of endogenously bound Na⁺ and K⁺ (1, 9). On the other hand, it is possible that the Na⁺-K⁺ ATPase activity is inhibited by the Pb²⁺ used in the Wachstein-Meisel medium. Other workers have also reported the inability to obtain Na⁺-K⁺ activation or ouabain inhibition in their histochemical preparations (12–14).

Our techniques have shown at least two nucleoside triphosphatase activities in the frog skin epidermis: one, in mitochondria, which is dependent on Mg²⁺, stimulated by dinitrophenol, and labile to fixation; and another, associated with cell membranes, which is not affected by dinitrophenol, inhibited by Mg²⁺, and reduced by fixation. Since divalent cations, especially Mg²⁺, are considered to be required for ATPase activity in general (18), we are placed in an apparently paradoxical situation of describing an enzyme which, instead of requiring Mg²⁺, is inhibited by it. As to the cause of this, the following speculation is offered. The inhibition of the cell membrane enzyme need not necessarily imply that Mg²⁺ is not required for its activity. Perhaps Mg²⁺ is more strongly bound to the cell membrane enzyme than to the mitochondrial enzyme and is not so easily dissociated in the media. The apparent inhibition due to the increased Mg²⁺ concentrations may indicate that the cell membrane enzyme requires the substrate to be chelated. In characterizing the "transport ATPase" in crab nerve, Skou (17) has noted that the Mg²⁺-ATPase was inhibited by relatively high concentrations of Mg²⁺, whereas the Na⁺-K⁺-activated Mg²⁺-ATPase was inhibited by relatively high concentrations of ATP. This Mg²⁺ inhibition and the likelihood of Pb²⁺ inhibition (3) of the Na⁺-K⁺ ATPase suggest that it is possibly the

¹K. T. Rogers. Personal communication.

Mg²⁺-ATPase (which may be an alternate activity of the "transport ATPase" (2)) which is demonstrated in histochemical preparations.

The strong chelation of ATP by Mg²⁺, reported by another worker (4), may lead to further complications of interpretations when one is using the Wachstein-Meisel medium. We have observed that, when Mg²⁺ is contained in the medium, after long standing a cloudiness appears. In media with only Pb²⁺ or Mg²⁺, little or no cloudiness was observed. The cloudiness was not due to lead phosphate deposits since our spectrophotometric analyses for the amount of inorganic phosphate were the same in media containing Pb²⁺ and Mg²⁺ or Pb²⁺ without Mg²⁺. These observations indicate that, in heterogeneous equilibria of the medium, unknown complexing is occurring and strongly indicate to us a need for a complete physicochemical analysis of the medium.

The fact that Mg²⁺ depressed the electrical potential difference, short-circuit current, and the nucleoside triphosphatase activity may be used to implicate more directly the cell membrane enzyme as part of the ion pump system. Also, it is tempting to link the observations that the membrane-bound enzyme was found localized histo-

chemically on the outer portions of the skin and that Mg²⁺ only inhibited the short-circuit current when it is applied to the outside and not to the inside of the skin. Caution should be exercised, however, in such an interpretation because, even though Mg²⁺ concentrations were the same in both the histochemical and bioelectric studies, the two series of studies were not comparable in respect to substrate concentrations. Furthermore, other workers (20) believe that Mg²⁺ does not penetrate the skin from the outside. While the drastic effects on electrical parameters by the addition of small amounts of Mg²⁺ cannot be attributed to slight changes in the osmolarity of the bathing solutions, the ions may have a target site totally different from the postulated transport enzyme in order to produce the large electrical effects.

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