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HYDROLYSIS OF EXOGENOUS ATP BY ISOLATED FROG GASTRIC MUCOSA

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SUMMARY

ATP, added to the solution bathing the nutrient (serosal) surface of isolated frog gastric mucosa, was found to break down rapidly to ADP, inorganic phosphate and other products. This activity is due to an ectoenzyme, i.e., to an enzyme system easily accessible to the bathing solution. This conclusion follows from experiments which showed that penetration of ATP into the mucosal cells occurred at a much slower rate: leakage of inorganic phosphate and adenine nucleotides from mucosal cells was also minor. The surface ATPase may reflect the operation of a mechanism at the nutrient surface involved in acid secretion.

INTRODUCTION

Various intact, isolated cells and tissues have been reported to catalyze the transformation of substrates added to the respective bathing media. Such enzymatic functions could prove useful in studying the properties of the plasma membrane, provided the substrate and products in the reaction can be shown to remain in the extracellular space. In that event catalysis can be attributed to an ecto-enzyme [1].

An approach of this kind would appear to be of value for gastric mucosa, in view of the controversy surrounding the site of origin of the several ATPases which have been obtained from that tissue [2]. Furthermore, attempts have been made to test the role of ATP as substrate for active transport in stomach, by looking for an effect of ATP added from outside [3, 4]. The presence of a surface ATPase can be expected to complicate the interpretation of such experiments.

The study describes an ATPase which is especially active when ATP is added to the nutrient (serosal) solution bathing the isolated gastric mucosa. Several approaches indicate that this ATPase resides on the cell surface. It is possible that the surface ATPase reflects the operation of a mechanism involved in active transport [5].

METHODS

The stomach was removed from a pithed bullfrog (*Rana catesbeiana*), the gastric mucosa freed of the outer layer of smooth muscle, and mounted between plastic chambers. The latter were 2.85 cm^2 in cross-section and contained either 6 ml (small chambers) or 3 ml (mini-chambers). The nutrient solution was in mM, 89.4 NaCl, 18 $NaHCO_3$, 4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 11 glucose and 0.1 histamine dihydrochloride, and was oxygenated with O_2–CO_2 (95 : 5 v/v): the secretory solution was 120 mM NaCl, oxygenated with 100 % O_2. The rate of acid secretion was followed by automatic buret and pH stat (Radiometer ABU 11 and TTT 1c), keeping the secretory pH at 7.7.

The mounted mucosa was allowed to recover for about an hour, at which time one of the bathing solutions was exchanged for a solution containing ATP. Unless otherwise noted, this was the nutrient solution with 0.5 mM ATP. Usually sampling was performed as follows: after a preincubation period of 10 min, the nutrient solution with ATP was renewed, and 1 ml samples taken at 1 and 11 min thereafter for measurement of inorganic phosphate (P_i). Thereafter, the sampling cycle was repeated, beginning each time with fresh solution containing ATP, but omitting the pretreatment. In a few experiments described in detail below, a different sampling procedure was used.

The ATPase activity was calculated as the net release of P_i in the bathing solution per minute. P_i was measured by the method of Lowry and Lopez [6]. In some experiments ATP, ADP and AMP were measured as well, using enzymatic, fluorometric techniques [7].

One group of experiments was performed with ATP[γ-^{32}P] in the nutrient solution at a level of 0.5 μCi/ml. The mucosa was mounted between Parafilm-Teflon gaskets to facilitate later dissassembly [8]. The concentration of ATP was reduced to 0.1 mM for easier measurement of changes in level, and the experimental period extended to 50 min for the same reason. Pre-incubation was omitted. Samples of the nutrient solution were taken at zero time (just after installation of labelled ATP), and at 10 and 50 min, for assay of total radioactivity and ATP level. At each of the above times, another aliquot of the nutrient solution was taken for separation of inorganic phosphate, using a modification of the Wahler-Wollenberger technique [9]. Total P_i was determined as usual in the upper phase, and both upper and lower phases were assayed for radioactivity. In this manner we could follow the increase in both radioactive and total inorganic phosphate during the incubation. At 50 min, either the gastric mucosa was removed, blotted rapidly and frozen in liquid Freon (cooled with liquid N_2), or the nutrient medium was exchanged twice in 1 min with non-labelled nutrient containing 0.1 mM ATP, and the mucosa removed, blotted and frozen. The tissue was weighed and pulverized while frozen [8], and the perchloric acid extract assayed for total radioactivity, as well as subjected to the Wahler-Wollenberger separation [9] to evaluate total and radioactive P_i. In a few experiments, the secretory solution was counted at the end of 50 min, to estimate passage of labelled phosphate across the mucosa.

For some experiments, isolated oxyntic cells were prepared by F. Michelangeli in our laboratory. The procedure is described fully elsewhere [10]. In brief, the isolated gastric mucosa was mounted on a plastic cylinder, and the luminal surface exposed to

a hypertonic salt solution to allow removal of the surface epithelial cells [11]. The glandular surface was then incubated with pronase, following the procedure of Blum et al. [12]. Detached cells were suspended in amphibian culture medium and stored at 4 °C for 1 to 2 days. Viability, as determined by exclusion of trypan blue, was always greater than 90 %; percentage of oxyntic cells, estimated by histochemical staining for succinic dehydrogenase, was about 82 %.

To measure ATPase, mucosal cells were suspended in 16 ml of a special nutrient solution, at a density of 10 to 15 mg (wet weight) of cells per ml. This solution contained (in mM) 70 NaCl, 4 KCl, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 40 *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid buffer, 11 glucose, 0.1 histamine diHCl; 1 % bovine albumin; and as antibiotics, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml). The suspension was stirred magnetically at room temperature (approx. 24 °C), and its pH was 7.2 when equilibrated with air.

At the beginning of the experiment, samples (2.2 ml) were taken 30 min apart to determine the spontaneous release of P_i. These samples, and those for P_i which followed, were immediately spun down in a refrigerated centrifuge for 5 min, to separate out the cells. The supernate was extracted with perchloric acid to remove albumin (which interfered with the P_i determination), and neutralized with $KHCO_3$; the final supernate was frozen for later P_i assay.

Following the determination of spontaneous P_i release, ATP was added to the cell suspension to a final concentration of 0.5 mM, and samples taken at 1 min, 16 min and 31 min thereafter to measure ATPase activity. Finally, [^{14}C]inulin was added to the remaining cell suspension at a level of 0.5 μCi/ml. Measured aliquots (2 ml) were removed at 3 min or 15 min thereafter, placed in weighed centrifuge tubes, and spun down as before. Samples of the supernate were saved for counting; the pellet was drained carefully and its wet weight determined, as well as its dry weight after drying to constant weight. The dried pellet was finally extracted with 0.1 N HCl for 24 to 48 h and the fluid counted to determine trapped volume (extracellular space). The latter did not appear to depend on the time during which the cells were exposed to the labelled inulin.

RESULTS

The hydrolysis of exogenous ATP is more rapid when ATP is added to the nutrient, rather than the secretory solution. This is illustrated in the experiment of Table I. Two halves (A and B) of the same mucosa were mounted in separate sets of chambers; after 30 min for recovery, spontaneous release of P_i was measured over the ensuing hour. ATP was then added to a final concentration of 1 mM either to the nutrient (A) or secretory (B) solution, and P_i release measured for 1 h in both solutions. The results indicate that ATP hydrolysis is much more vigorous when ATP is present in the nutrient solution: in addition, it is clear that P_i release into the solution without ATP can be accounted for by release of endogenous P_i.

The rest of the experiments to be reported here characterize further the hydrolysis of ATP when it is added to the nutrient solution.

To determine the extent to which ATP was broken down, we performed experiments with the mucosa using minichambers (3 ml in volume). The spontaneous leakage of ATP, ADP, AMP and P_i was first measured over a control period of 1 h, then a

TABLE I

HYDROLYSIS OF ATP IN NUTRIENT OR SECRETORY SOLUTION

In two mounted halves (A and B) of a gastric mucosa, spontaneous release of P_i was first measured for 1 h in the absence of external ATP: ATP was then added at a level of 1 mM to the nutrient (A) or the secretory (B) solution, and P_i release measured for the ensuing hour.

	[ATP] (mM)	P_i release (nMol/h)	
		Into nutrient solution	Into secretory solution
(A) In nutrient	0	59	39
	1	4340	32
(B) In secretory	0	52	72
	1	36	216

nutrient solution containing 0.5 mM ATP was introduced and assayed for nucleotides and P_i at the end of 30 min. The results are summarized in Table II. Except for ADP, release of nucleotides and P_i during the control period was minor in comparison to the changes occurring with ATP present. The ratio of P_i liberated to ATP hydrolyzed was 2.2, a value similar to that measured for intact yeast cells [13] and skeletal muscle [14]. The excess of ATP hydrolysis over ADP and AMP released indicates that adenosine or inosine has been formed [4]. Assuming that is the case, we can draw up a balance sheet for phosphate (Table II B). The calculated phosphate split from ATP agrees reasonably well with the P_i measured in the nutrient solution, supporting the view that the reactions involved occur at the mucosal surface.

The ATPase activity is quite reproducible at a given ATP level, despite frequent changes of nutrient, as will be seen later in Fig. 3. Thus it did not seem likely that the

TABLE II

PRODUCTS OF HYDROLYSIS WITH ATP IN NUTRIENT SOLUTION

Part A shows the spontaneous release of nucleotides and P_i into the nutrient solution, compared with changes occurring in the presence of ATP, initially at a level of 0.5 mM: pooled results for 2 mucosae. In part B, a balance sheet is drawn up to list sources of P_i.

(A) Condition	Release (nmol/h)			
	ATP	ADP	AMP	P_i
control	1	59	0	18
0.5 mM ATP	−975	423	317	2179
net	−976	364	317	2161

(B) Source	P_i calculated (nmol/h)
ADP	364
AMP	634
adenosine etc.*	885
Sum	1883

* Calculated as 3 [976−(364+317)] = 3 (295) = 885.

TABLE III

ASSAY FOR RELEASED ATPase

In part A, P_i release was measured in a beaker, using nutrient solution which had bathed gastric mucosa for 10 min, in part B, the nutrient solution was in contact with the gastric mucosa while P_i release was measured. Data are for two gastric mucosae, number of measurements are in parentheses.

Condition	[ATP] (mM)	ATPase (nmol P_i/min)
(A) preincubated solution (4)	0.5	2.6
(B) contact (2)	0.5	47.2

ATPase had a trivial origin, due to bacteria, enzyme released from damaged cells, etc. Nevertheless we considered it worthwhile to make a direct test. A nutrient solution (free of ATP) which had been in a chamber in contact with a mucosa for 10 min, was brought to a final ATP level of 0.5 mM and incubated 10 min in a beaker, oxygenating with the usual mixture of O_2 and CO_2. Samples were taken at the beginning and end of this period for P_i assay. Table III compares the activity thus obtained with the activity measured for the same mucosa with ATP present in the nutrient solution in contact with the mucosal surface. The latter is greater by far, hence not due to enzymes separated from the mucosa.

Hydrolysis of ATP by the mucosa could reflect surface activity, or be due to intracellular enzymes, provided substrate and products pass freely across cell membranes. To distinguish between these possibilities, we performed a series of experiments with ATP [γ-^{32}P].

The nutrient solution contained at the outset, 0.1 mM ATP and approx. 3 μC ATP[γ-^{32}P] in 6 ml. Analysis of the nutrient after 50 min of incubation (cf. Methods) showed that 32.7$\pm$2.3 % (S.E.M., 6 exp) of the initial ATP had been hydrolyzed to radioactive, inorganic phosphate. This figure conformed well to the estimate of ATP breakdown by direct fluorometric analysis (33$\pm$3 %).

Nearly all the labelled ATP and P_i remained in the nutrient solution, as judged by the assay of total radioactivity (cpm/ml): at the end of 50 min, this was 97.5$\pm$0.3 %

TABLE IV

SPECIFIC ACTIVITIES

Results of 4 experiments with ATP[γ-^{32}P] placed in the nutrient solution, comparing the initial specific activity of ATP with the specific activity of inorganic phosphate in the nutrient solution and tissue after a 50-min incubation.

Exp.	No. of washes	Spec. act. (cpm/nmol)		
		Initial nutrient ATP	Final nutrient P_i	Final tissue P_i
2/20 a	0	9600	3330	186
2/20 b	2	9600	3720	137
3/5 a	0	13510	4490	225
3/5 b	2	13510	3797	140

of the initial value in 6 exp. During this time, 0.01 % of the initial radioactivity passed over to the secretory solution (4 expts).

These results indicate that the final tissue radioactivity could not have exceeded a few percent of that initially added to the nutrient, and this was confirmed by counting tissue organic and inorganic phosphate. The Wahler-Wollenberger separation yielded data for calculating the specific activity of P_i in the tissue and nutrient solution at the end of the 50 min incubation, and these values are given in Table IV.

The final specific activity for P_i in the nutrient is less than the initial value for ATP, due to dilution by unlabelled P_i from ATP hydrolysis. As expected, the double wash reduced the specific activity of P_i in the tissue, probably by extracting labelled P_i from extracellular space. Whether or not the tissue was washed, the final specific activity of P_i in the tissue was smaller by an order of magnitude than that of P_i in the nutrient solution. This result excludes the possibility that nutrient P_i had originated primarily from entry of ATP into the mucosal cells, followed by intracellular hydrolysis and diffusion of P_i from the cells. Instead it appears that the major fraction of ATP hydrolysis occurred on the cell surface (or in a small region easily accessible to the surface). It is apparent, however, that a small amount of ATP or P_i did enter the cells during the incubation.

The isolated gastric mucosa contains several cell types which could be responsible for the surface ATPase. To obtain further information concerning its locus, we turned to the isolated cell preparation (cf. Methods). The latter contains approx. 82 % oxyntic cells and is virtually free of connective tissue and smooth muscle.

Isolated cells were suspended in a conventional medium (cf. Methods) and allowed to equilibrate for 10–15 min. Samples were taken over the following 30 min to assay release of P_i in the absence of ATP; ATP was then added to a final concentration of 0.5 mM, and sampling continued for the ensuing 30 min. In 4 different batches of cells, we found an ATPase activity of 0.81 ± 0.15 (S.E.M.) nmol P_i per min per mg weight of wet cells. The latter has been corrected for inulin space, and for the spontaneous release of P_i, which amounted to 0.09 ± 0.06 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ cells. In 2 experiments, we attempted to quantitate spontaneous release of ATP during the initial period (no exogenous ATP), but it was too small to be measured. The cell content of ATP was easily determined, however, amounting to 3.0 nmol per mg wet weight of cells in 2 experiments.

The activity of surface ATPase in the isolated mucosa is about 40 nmol P_i/min at an external [ATP] of 0.5 mM (cf. Tables II and III). Since a mucosa as used here. has a wet weight of about 0.2 g, we may infer that the surface ATPase of intact mucosa is approx. 0.2 nmol $min^{-1} \cdot mg^{-1}$, i.e. about a quarter of that of the isolated cells. Of course the figures are not directly comparable: cells in the intact mucosa may be less accessible to external ATP, and the mucosal weight has not been corrected for non-oxyntic cell mass. Nevertheless, it appears that much (and perhaps most) of the surface ATPase activity is located on oxyntic cells.

The remainder of this section deals with the dependence of the nutrient surface ATPase on nutrient pH and concentration of substrate. Both of these factors affect acid secretion, and these experiments therefore introduce the question of the relationship of the nutrient surface ATPase to the secretory process.

In examining the effect of nutrient pH, we substituted 18 mM Tris for the 18 mM $NaHCO_3$ of the usual nutrient solution, and replaced the 95 : 5 mixture of O_2

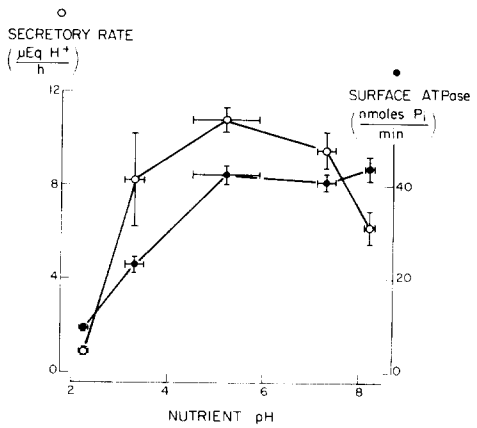

Fig. 1. Dependence of acid secretion and surface ATPase on pH of nutrient solution: pooled results of 12 expts. Experimental procedure is described in the caption of Fig. 2.

and CO_2 with 100% O_2. Aliquots of the Tris-nutrient were adjusted with HCl to values of pH between 2.3 and 8.1. While the buffer had little effect over much of that range, the procedure had the advantage of simplicity. The nutrient pH was monitored during the experiments and it remained quite stable during the 10–11 min required for an ATPase measurement, provided the pH was near the high or the low extreme. In between, the nutrient pH increased due to extrusion of alkali as a consequence of acid secretion. This shift, together with a slight variation in solution pH among experiments, is shown as a horizontal bar in the cumulative results displayed in Fig. 1.

The results indicate a single broad maximum for both surface ATPase and acid secretion as a function of pH. The deleterious effect on acid secretion of a nutrient pH less than 3 agrees with the prior finding of Sanders et al. [15]. Low nutrient pH

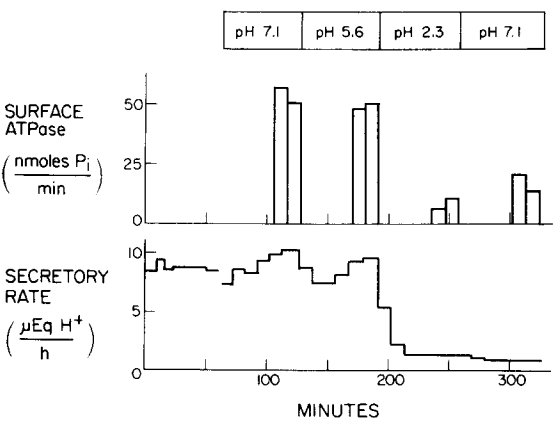

Fig. 2. Irreversible damage due to nutrient solution of pH 2.3. This figure illustrates the procedure used in pH experiments; the mucosa was exposed to a nutrient solution with 0.5 mM ATP at the given pH for 66 min (six 11-min periods). During the last 2 periods with each pH, surface ATPase was measured in the usual manner.

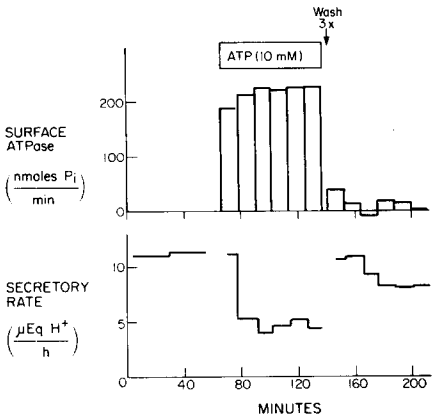

Fig. 3. Surface ATPase at the level of 10 mM ATP, and the effect of this concentration on acid secretion. ATP was present for six 11-min periods in sequence, as shown; following its removal, the nutrient solution was sampled for six 11-min periods to assay for delayed release of P_i.

produced irreversible damage to both the acid-secreting mechanism and surface ATPase, as illustrated in Fig. 2. While some ATPase activity was restored, the final rate at pH 7.1 following an exposure to pH 2.3 for 1 h was 52 ± 8 % of the initial activity at pH 7.1, in 5 exp. This inhibition was significant ($P < 0.005$). In contrast, inhibition of ATPase by a nutrient pH of 3.2 was completely reversible (4 expts).

It has been previously reported that ATP, added to the nutrient solution at a sufficiently high level, can inhibit acid secretion [3, 16]. The results presented thus far suggest that such exogenous ATP will be hydrolyzed at a rapid rate, and this is the case, as illustrated in the experiment of Fig. 3. The nutrient containing 10 mM ATP was titrated to the usual nutrient pH and the osmolarity adjusted to the normal value by removing NaCl; this solution was instilled during six consecutive periods of 11 min

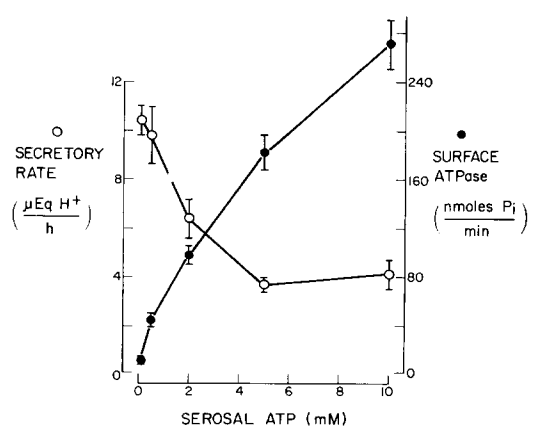

Fig. 4. Dependence of surface ATPase and acid secretion on level of ATP in nutrient solution. As in pH experiments, an individual result represents the average of the last 2 periods of a six-period sequence at a given ATP level. The means plotted are derived from 14 expts.

each. Fig. 3 shows that the surface ATPase reached a plateau value rapidly, with inhibition of acid secretion occurring soon thereafter. Removal of ATP was followed quickly by a drop in P_i release to essentially zero, and recovery of normal acid secretion. Three other experiments gave similar results.

In a final group of experiments, intermediate concentrations of exogenous ATP were examined. Several levels of ATP were tested in each mucosa, and the results pooled in Fig. 4. Inhibition of acid secretion did not become severe until levels of 2 mM or greater were reached. The surface ATPase rose with increasing ATP level but failed to reach the well-defined plateau characteristic of Michaelis-Menten kinetics (cf. Discussion).

DISCUSSION

The isolated gastric mucosa rapidly catalyzes the breakdown of ATP placed in the nutrient bathing solution, as first reported by Kidder [4]. His results, with the present studies, show that the products of hydrolysis include ADP, AMP, adenosine, P_i and various inosine compounds.

Where does this process occur? If external ATP readily enters oxyntic cells and is utilized there, it should drive active transport under circumstances in which endogenous ATP is rate-limiting, for example, during hypoxia. Kidder could not find any such effect, and concluded that ATP was not the immediate substrate for acid secretion [4].

The assumption that the vigorous hydrolysis of external ATP reflected its rapid entry into oxyntic cells can be rejected if hydrolysis occurs principally at the cell surface. The latter alternative is supported by a number of findings in the present study. (1) Enough products, including P_i, appeared in the nutrient solution to account for the ATP hydrolyzed: spontaneous leakage of internal P_i and adenine nucleotides, on the other hand, was relatively trivial. (2) The release of P_i into the nutrient reached a plateau within a few minutes after addition of external ATP, and disappeared as rapidly upon removal of ATP. (3) When ATP[γ-^{32}P] was included in the nutrient solution, sufficient $^{32}P_i$ appeared in that solution to account for the ATP hydrolyzed. At the end of 50 min of exposure, the specific activity of mucosal P_i was much less than that of the nutrient; thus the latter did not arise from diffusion of labelled P_i from the cells.

A small fraction of the ATP added to the nutrient solution eventually penetrates into the mucosa cells, however. A crude upper limit for the rate of entry can be obtained from the present results. Table IV shows that, at the end of 50 min, the specific activity of P_i in the mucosa is 139/3759, = 1/27, of the nutrient P_i. The concentration of P_i is approximately 1.6 μmol/g wet weight, as determined by Forte et al. [17] and by us (unpublished results); the wet weight of a mucosa as used here is roughly 0.2 g, so that (1/27)(1.6)(0.2) or about 10^{-2} μmoles of tissue P_i became labelled during the incubation. We cannot tell whether this represents entry of labelled P_i, or entry of AT^{32}P followed by intracellular hydrolysis. The Wahler-Wollenberger separation used in this study showed that the tissue contents of labelled organic and inorganic phosphate were comparable (unpublished results). Hence an upper limit for the total entry of labelled ATP would be twice the rate given above, or 20 nmol/50 min = 0.4 nmol/min. This figure is valid for the initial ATP concentration of

0.1 mM we used. If entry were linear with external ATP, it would rise to 40 nmol/min at the initial external ATP level of 10 mM used by Kidder [4]. The latter quotes an entry rate of 0.38 μmol · cm^2 · h^{-1}, which would amount to 18 nmols · min^{-1} for the mucosal area of 2.85 cm^2 used in this study. Thus our results are in rough agreement with those reported by Kidder.

Why then does not external ATP support acid secretion during hypoxia? The answer here is probably complex; ATP is likely to be degraded in the process of entry; large concentrations of ATP ($\geqslant$ 2 mM) are in themselves inhibitory; hypoxia may deplete the mucosa of necessary constituents other than ATP.

The finding that isolated mucosal cells had considerable ATPase activity led us to conclude that some, and perhaps all, of the nutrient surface ATPase is localized to the oxyntic cells. This conclusion is based on the assumption that the isolated cells, like cells in the intact mucosa, were relatively impermeable to ATP. In support of this belief, we found that leakage of endogenous P_i and ATP from the isolated cell preparation was small compared to the surface ATPase measured at 0.5 mM. In addition, the isolated cells respire at a steady rate and maintain normal levels of ATP, ADP, creatine phosphate and creatine for many hours [10].

The finding here that a moderate reduction of nutrient pH can increase acid secretion agrees with the previous results of Sanders et al. [15]. In the absence of external CO_2, the increased acidity of the nutrient diffusion barrier serves to trap HCO_3^- produced by the oxyntic cells during acid secretion. The resultant CO_2 is formed sufficiently close to those cells so as to be available for buffering of intracellular OH^-. Thus the pH of interstitial fluid near the oxyntic cells is undoubtedly much higher than the nutrient pH, at least when the latter is reduced much below neutrality.

The nutrient diffusion barrier also must be taken into account when the dependence of surface ATPase on nutrient ATP levels is considered (Fig. 4). The ATP level at the site of hydrolysis will be less than that in the external medium, so that the diffusion down this gradient will just equal the rate of hydrolysis. This is the situation analyzed by Winne [18], who showed that calculation of the Michaelis constant, K_m, from curves such as that of Fig. 4 requires a determination of the thickness of the unstirred layer.

Studies with chelating agents show that the nutrient surface ATPase is activated by Mg^{2+} [5]. Thus this activity may coincide with that reported by Koenig and Vial, who used a histochemical method at the electronmicroscopic level to identify a Mg^{2+}-activated ATPase on the basal-lateral surfaces of toad oxyntic cells [19]. In addition, these authors found that the reaction product (lead phosphate) appeared to be present on the outside of the cell membranes.

Some evidence points to a relationship between the nutrient surface ATPase and acid secretion. We saw in the present study that low nutrient pH (< 3) inhibited irreversibly both the ATPase and acid production: other studies [5] showed that various agents added to the nutrient solution inhibited both types of activity. These agents included fluoride ion, *p*-chloromercuribenzene sulfonate and EDTA. Furthermore, the action of large concentrations of external ATP to inhibit acid secretion may reflect some kind of competition at a site accessible to ATP from inside or outside the oxyntic cell.

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