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THE ENERGY SOURCE FOR GASTRIC H^+ SECRETION

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SUMMARY

Acid rate, resistance, short circuit current, P.D., Cl^- transport, O_2 consumption and redox steady state were determined in frog or Necturus gastric mucosa under a variety of conditions. It was shown that measurements of $\Delta H/\Delta O_2$ under conditions of stimulation of acid rate by hormones in Necturus, or ion substitution in frog did not allow valid conclusions to be drawn as to acid mechanism. The simplest interpretation of the results of a series of experiments using redox inhibitors and phosphorylative inhibitors and uncouplers is that mitochondrially generated ATP is the obligatory energy source for H^+ transport, and that Cl^- transport can utilize extramitochondrial sources of energy.

INTRODUCTION

Two sources of energy for H^+ transport have been considered in the stomach, redox energy or ATP and the chemical intermediates involved in ATP synthesis. The redox theory[1] as originally proposed postulates a direct transfer of H^+ across the secretory membrane, with the H^+ directly evolved from substrate, *viz.*

$$AH_2 + {}^1/_2\,O_2 \rightarrow A + 2H^+ + {}^1/_2\,O_2{}^{2-}$$

It is clear from this that the maximal H^+/O_2 ratio cannot exceed 4, and no laboratory has shown ratios greater than 4, most of the values reported falling in the range 2–3. These studies in recent years have been carried out mainly on amphibian mucosa[2]. However, since parietal cells comprise only 20 % of the cell population of the chambered frog gastric mucosa, and the fractional metabolism of these cells related to acid secretion is unknown, this overall ratio is probably an underestimate. An alternate approach has been to relate changes in acid rate with changes in O_2 consumption ($\Delta H/\Delta O_2$) with the use of inhibitors, either indirect such as amytal or presumably direct such as SCN^- (ref. 3).

The significance of the $\Delta H/\Delta O_2$ ratio depends on several assumptions relating not only to specificity of inhibition (it is evident that inhibitors which virtually abolish O_2 consumption will give values similar to the H^+/O_2 ratio discussed above, provided H^+ is inhibited) but also to mechanism of action on the transport process. Thus if SCN^-, which gives large $\Delta H/\Delta O_2$ ratios, incompatible superficially with a simple redox mechanism, acts not by interfering with the energy transfer reaction,

but with one of the latter stages of transport, for example dissociation of H^+ from the carrier on the luminal surface of the cell, then high $\Delta H^+/\Delta O_2$ values would not relate to presence or absence of a redox mechanism. Stated alternatively SCN^- could be acting as an "uncoupler" of transport, hence in analogy to the well known uncouplers of mitochondrial energy metabolism, O_2 consumption would be virtually unaffected by SCN^-.

From the preceding discussion therefore one cannot decide from measurements of H rates and O_2 consumption as to whether an ATP or redox driven mechanism is operative.

Three other approaches have been used in this work. Firstly, one can attempt without the use of inhibitors to alter H^+ rates, either by stimulation of secretion, or by ion substitution. This approach may also be subject to a variety of interpretations. Secondly, one can attempt to correlate changes in redox components with changes in transport, assuming that the redox component (if any) related to H^+ secretion is observable by the given technique. Finally one can use inhibitors of the various cellular reactions, particularly mitochondrial, to attempt to elucidate the critical reactions required for transport. The evident argument against the use of inhibitors rests on the unknown specificity of most of these compounds in intact tissue. However, by judicious selection of concentration and by use of an adequate number of inhibitors for a given reaction or reaction sequence, the problem of non-specificity is minimized. It is also necessary to measure as many metabolic parameters as is justifiable to ensure that there is no gross lack of specificity in the action of any individual inhibitor.

We therefore investigated: (a) the action of histamine, mecholyl or pentagastrin* on Necturus gastric mucosal O_2 consumption and acid rate, since this tissue is sensitive to those compounds *in vitro*[4]; (b) the effect of substituting Cl^- for SO_4^{2-} in the bathing media of frog gastric mucosa since higher acid rates are obtained by this means[5]; (c) the correlation between changes in transport and redox changes; and (d) the effects of a variety of redox and phosphorylative inhibitors and uncouplers on gastric mucosal metabolism and transport.

METHODS

Frog (*Rana pipiens*) or Necturus gastric mucosa was stripped of muscle layer and mounted in an Ussing chamber by previously described procedures. The measurement of P.D., resistance, short circuit current (I_{sc}), Cl^- flux and H^+ rate has been previously outlined[6]. Determination of redox components was carried out in an Aminco Chance dual beam spectrophotometer[7] and O_2 measurements with a Clark electrode in a Kel-F chamber with magnetic stirring[8] using a Radiometer system. Water used was double glass distilled, and chemicals were of the highest grade available. In anion substitution experiments, SO_4^{2-} was substituted for Cl^-, and all chemicals were added to the nutrient side after a control period varying between 30 min and 1 h. The phosphorylative inhibitors used were gifts from Dr. H. A. LARDY.

* ICI 50, 123.

RESULTS

Stimulation of acid secretion

The fact that Necturus gastric mucosa is usually obtained in a resting state and can be stimulated with respect to acid secretion[4] allowed experiments to be performed on the effect of mecholyl (10^{-7} M), histamine (10^{-4} M) and pentagastrin (10^{-9} M) on O_2 consumption in addition to effects on H^+ rate, P.D., I_{sc} and resistance.

After addition of histamine O_2 consumption rises slightly approx. 30 min following the onset of secretion (Fig. 1). The rise in P.D., with fall of resistance, and hence rise in I_{sc} is typical of increased Cl^- transport, and not of initiation of a primary H^+ electrogenic pump[9]. Pentagastrin shows a similar effect.

Fig. 2 shows the effect of mecholyl. The spike in the P.D. appears to be associated with a rise in O_2 consumption and we have shown this P.D. spike to be related to Cl^- transport. With onset of acid secretion, however, there is no further increase in O_2 consumption.

Cl^-–SO_4^{2-} substitution: Frog gastric mucosa will secrete H^+ in both Cl^- or SO_4^{2-} solution, although the rate is 2–3 fold greater in Cl^- compared to SO_4^{2-}. Furthermore Cl^- is actively transported by this tissue[10] whereas SO_4^{2-} is not. Thus it might be anticipated that changes of bathing solution would significantly affect O_2 utilization of the tissue. Fig. 3 shows such an experiment in which Cl^- was substituted for SO_4^{2-}. Although significant changes were obtained in P.D., I_{sc} and H^+ rate, O_2 consumption remained steady.

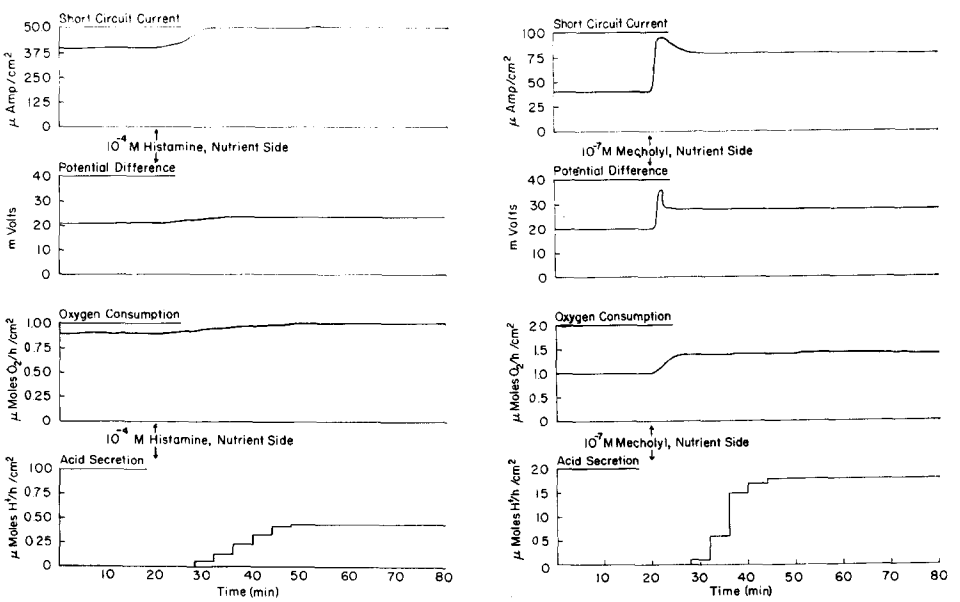


Fig. 1. Effect of 10^{-4} M histamine added to the nutrient side of Necturus mucosa *in vitro* on short circuit current, P.D., acid rate and O_2 consumption.

Fig. 2. Effect of 10^{-7} M mecholyl on nutrient side of Necturus gastric mucosa, showing the spike obtained in the P.D. with the corresponding rise in O_2 consumption occurring prior to the onset of acid secretion.

Redox changes

Frog gastric mucosa transports both H^+ and Cl^- aerobically. With anaerobiosis, however, H^+ secretion is rapidly inhibited (within 5 min), and there is maintained short circuit current or ^{36}Cl flux for a period of 40 min following H^+ inhibition. With H^+ inhibition there is also reduction of the redox components of the respiratory chain. No correlation exists between the degree of H^+ inhibition and that of reduction of the redox chain[18].

Inhibitors

The inhibitors used, with their presumed locus of action are shown in Table I. The experimental procedure was to use the O_2 consumption chamber, and add increasing concentrations of the inhibitor on the nutrient side until an effect was obtained. Certain of the compounds were dissolved in propylene glycol before addition, or sonicated into suspension in nutrient medium prior to addition.

Substrate level inhibition: Neither malonate or oxamate at concentrations up to 10^{-2} M had any effect under aerobic conditions. Anaerobically, oxamate resulted in some depression of the P.D. It is likely that poor penetration can account for these data. 2-Deoxyglucose has been shown to inhibit acid secretion, and at concentrations greater than 10 mM, also to affect the I_{sc} (ref. 6).

Redox inhibitors: These inhibitors show qualitatively similar effects on frog gastric mucosa. Of the compounds tested, only rotenone had no effect, presumably

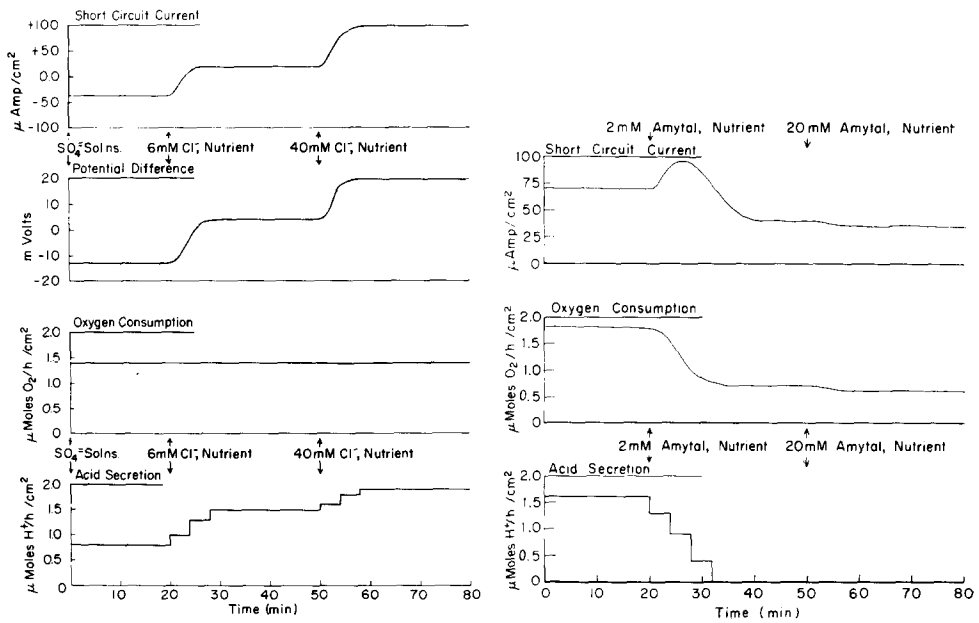


Fig. 3. Effect of serial addition of Cl^- (6 mM, 40 mM) to nutrient side of *R. pipiens* gastric mucosa showing a steady O_2 rate in the face of changing I_{sc} and H^+ rate.

Fig. 4. Amytal at 2 and 20 mM added to *R. pipiens* gastric mucosa with measurement of I_{sc}, O_2 rate and H^+ rate.

TABLE I

COMPOUNDS TESTED IN GASTRIC MUCOSA

Given are minimal effective concentrations (mM) at 15 min following addition. (—), no effect.

Substrate level inhibition	*Redox inhibitors*	*Redox acceptors*	*Phosphorylative uncouplers*	*Phosphorylative inhibitors*
Malonate (—)	Amytal (2)	Phenazine methosulfate	Dinitrophenol (0.05)	Oligomycin (—)
Oxamate (—)	Rotenone (—)	Methylene blue	Pentachlorophenol (0.03)	Rutamycin (—)
2 Deoxyglycose (10)	Antimycin A (0.05)	Tetramethyl-*p*-phenylene diamine	Arsenate (2)	Aurovertin*
	Thenoyl trifluoroacetophenone (0.1)	Ferricyanide	*m*-Chlorocyanocarbonyl phenyl hydrazone (0.001)	Atractyloside (0.01)
	Azide (1)		1,1,3-Tricyano-2-amino-1-propene (0.01)	Galegine sulfate (1)
	Cyanide (1)			Phenethyl biguanide (0.1)

* Concentration 3 μg per mg tissue.

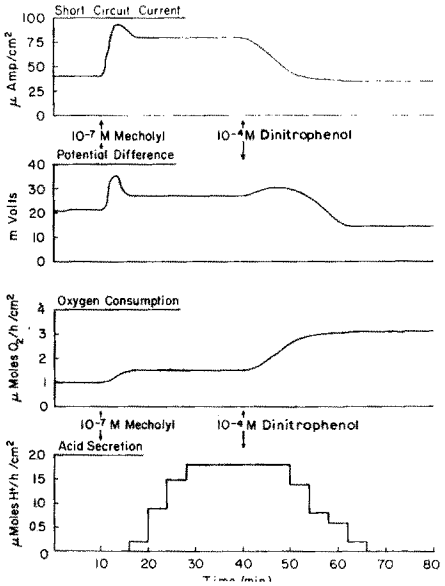


Fig. 5. Effect of dinitrophenol (10^{-4} M) on Necturus gastric mucosa, illustrating the fall in I_{sc}, H^+ rate and P.D. with rise in O_2 consumption.

due to non-penetration of the tissue. Inhibition by these compounds leads to a rapid fall of acid rate and a sustained increase in resistance. There is a transient rise in I_{sc}, followed by a fall to a lower, but sustained, level, which can be reversibly inhibited by anoxia. Fig. 4 illustrates the action of amytal at 2 mM followed by 20 mM. A simultaneous plot of ATP levels show a reduction in ATP between control and 2 mM, but only a slight further reduction at 20 mM amytal. Crossover points were established and were typical of those found in isolated mammalian mitochondria. These redox changes have been previously detailed[18]. The inhibition of cyanide was atypical, however, in that high concentrations were required for an effect to be seen, of the order of 10^{-3} M and at this level the effect often was spontaneously reversible with acid, I_{sc} and even to the point of reoxidation of cytochrome a_3. At 10^{-2} M no such spontaneous reversal was obtained*.

Redox acceptors: Since electron flow in mitochondria can be reestablished by suitable redox acceptors, certain dyes were used in an attempt to reverse the transport inhibition. Various combinations of β-hydroxybutyrate, succinate, ascorbate, amytal, antimycin A and cyanide with the dyes listed in Table I were tried, both in O_2 and N_2 gassed mucosae, and although reduction of the dye was obtained, no H^+ secretion was obtained. It may be assumed therefore that electron flow was reestablished, without significant ATP synthesis. With tetramethylene-*p*-phenylene diamine, an artifact was obtained due to pH dependent dye transport. When this was eliminated by suitable adjustment of the pH, no acid rate was obtained.

Action of phosphorylative uncouplers

The effects of dinitrophenol on the various parameters of the frog gastric

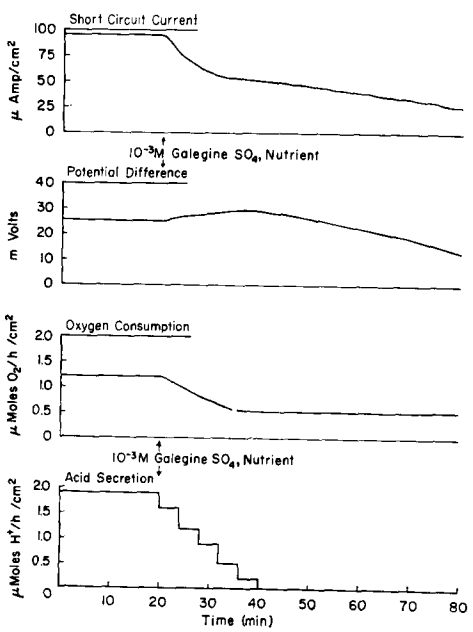


Fig. 6. Effect of 10^{-3} M galegine sulfate on O_2, P.D., I_{sc} and acid rate in *R. pipiens* gastric mucosa, showing maintenance I_{sc} with absent H^+ rate.

* W. S. REHM AND G. SACHS, unpublished results.

mucosa have been well described[11]. However most of the other uncouplers have not been tested, and Fig. 5 shows the typical effect of an uncoupler (in this case dinitrophenol) on acid rate, P.D., I_{sc} and O_2 consumption of Necturus gastric mucosa. Again, it is typical of this group of compounds that I_{sc} can be maintained in the face of complete H^+ inhibition. All the chemicals listed in Table I were effective, with *m*-chlorocyanophenyl hydrazone at 10^{-6} M being the most active.

Phosphorylative inhibitors

The effects of these compounds on the tissue was variable. Oligomycin and rutamycin had no detectable action on any of the parameters. Various modes of addition, including suspension in dimethyl sulfoxide were ineffective. However site-specific inhibitors such as phenethyl biguanide and galegine sulfate blocked transport and O_2 consumption (Fig. 6). In addition, aurovertin inhibited acid secretion along with O_2 rate (Fig. 7). Addition of atractylate (Fig. 8) at high concentrations also blocked secretion and O_2 consumption. Once again, I_{sc} was less sensitive to these inhibitors than H^+ rate.

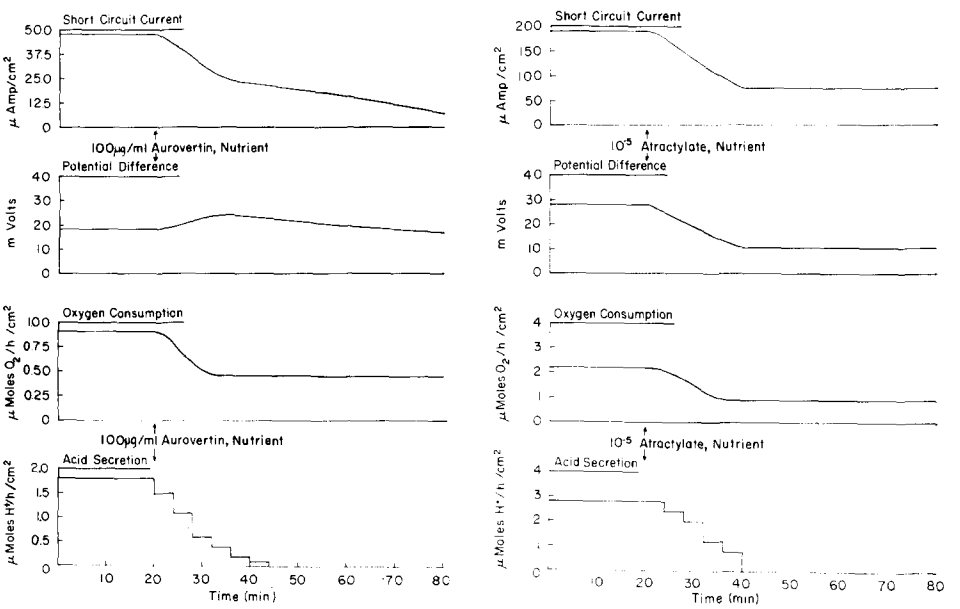


Fig. 7. Action of 100 μg/ml aurovertin on Necturus gastric mucosa with inhibition of acid rate, O_2 consumption, maintenance of P.D. and slow fall of I_{sc}.

Fig. 8. Action of atractylate at 10^{-5} M on *R. pipiens* gastric mucosa with changes typical of the group of phosphorylative inhibitors.

DISCUSSION

In considering the experiments where acid rates were changed by the use of stimulants of acid secretion, although there was a rise in O_2 consumption, the details of the results were unexpected. Thus, in the case of histamine and gastrin stimulation, the rise in O_2 was slight and prolonged rather than rapid, started before the onset of

acid, and continued to rise during the phase of rising acid rate without any observable change in slope. The ratios obtained when we considered the overall change in rate of oxygen utilization relative to the acid rate were of the order of 4 or greater, but if we calculated the changes from the time of onset of acid rate, the values obtained were 6 or more. It is this latter calculation which would be more pertinent to an assessment of redox theory since altered rates of H^+ transport would be reflected in simultaneous and stoichiometric changes in O_2 consumption. The only alternative to this view is that redox secretion channels consumption normally reserved for phosphorylation into transport, thus effectively uncoupling the redox chain at one or more sites. This will be discussed in greater detail later.

The action of mecholyl further substantiates the data obtained with histamine and gastrin, in that, although there was a rapid rise of O_2 rate this was related to the P.D. spike observed with this stimulant, and consumption remained steady during the onset of acid secretion. Hence with this compound ratios of H^+ rate changes to oxygen rate changes were close to infinite. Since the P.D. spike is related to stimulation of Cl^- transport, apparently transport of this ion is involved in the oxygen changes seen, and in reexamining the histamine and pentagastrin data, the gradual change observed is also related to changes in I_{sc} rather than acid rate.

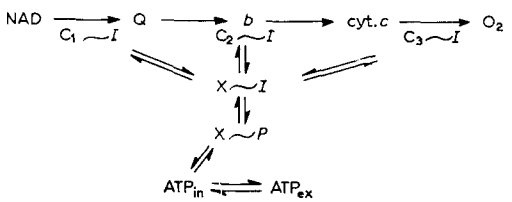
Similar results are obtained when Cl^- is substituted for SO_4^{2-} in the bathing media of frog gastric mucosa. In this type of experiment, both H^+ and anion transport increase several fold upon the addition of Cl^- to the bathing medium. Thus acid rate increases 2–3 fold and I_{sc} increased by about 4 μequiv/h. In spite of these significant changes in transport, there is no change in O_2 utilization. This is very reminiscent of SCN experiments where little change is seen during reduction of acid rate. These data confirm observations made in a similar manner using glucuronate solutions instead of SO_4^{2-} (ref. 12).

It appears therefore, both in resting Necturus and in frog mucosa bathed with non-transportable anion, that stimulation of transport either diverts energy from other processes into transport function, or in some way couples a previously uncoupled transport system. In either case, measurements of $\Delta H/\Delta O_2$ ratios yield little useful information.

The mitochondrial redox system can function independently of phosphorylation only in the uncoupled state. If the redox theory held then the energy available would be utilized for transport, and not phosphorylation. Thus redox theory implies uncoupling of the phosphorylative mechanism. Both theories would be expected to show sensitivity to redox inhibitors, and in the case of redox theory, electron acceptors inserted on the substrate side of the inhibition might be expected to reverse inhibition of acid secretion by redox inhibitors if a redox mechanism were operating. This was not found to be the case. Furthermore the sensitivity of H^+ transport to redox inhibition (including anoxia) shows that oxidative reactions are critical; and also that these reactions are mitochondrial in nature, since classical mitochondrial inhibitors are effective at concentrations which do not influence non-mitochondrial oxidations.

The relative insensitivity of the Cl^- mechanism to anoxia and other forms of redox inhibition suggests that the energy for anion transport may be derived by anaerobic metabolism such as glycolysis.

A current view of the chemical mechanism of oxidative phosphorylation is as follows[13]:



In this representation 3 sites of the chain show limited phosphorylation represented by $C_1 \sim I$ *etc.* Dinitrophenol and other uncouplers may be considered to prevent interaction between these compounds and the redox chain by promoting hydrolysis of these intermediates. Galegine sulfate on the other hand is an inhibitor of phosphorylation presumably acting by inhibition of formation of $C_1 \sim I$, without accompanying hydrolysis. Phenethyl biguanide similarly interacts between the chain and $C_2 \sim I$. These are then site-specific inhibitors[14].

$X \sim I$, the next compound postulated, is responsible for the various ion translocation properties of mitochondria and is affected by antibiotics such as valinomycin, nonactin, *etc.* Hence ion transport in this scheme uncouples respiration from ATP synthesis. Oligomycin is considered to act at the reaction where phosphorolysis of $X \sim I$ occurs[15], and aurovertin between $X \sim P$ and ATP (ref. 16). Atractyloside then inhibits transfer of energy between internal ATP and external ADP (ref. 17).

The action of uncouplers in inhibiting acid secretion may be interpreted as evidence for the involvement of ATP, or the intermediates of phosphorylation in H^+ transport, since as discussed above, a redox mechanism for secretion implies uncoupling of the chain. Since all uncouplers show this effect, we may also conclude that the energy source for acid secretion is mitochondrial. Since uncouplers act on the initial stage of phosphorylation, subsequent to H^+ and electron transfer, the only form of redox mechanism which is tenable would be one where both redox energy and product of the phosphorylating chain would be required.

Site-specific inhibitors such as phenethyl biguanide or galegine sulfate inhibit acid and O_2 consumption in the stomach. This would imply that respiration is tightly coupled in the tissue in contrast to what might be expected if a redox mechanism were operating. These data then suggest that ATP or an intermediate is responsible for acid secretion. If one still holds to redox theory then the effect of site I inhibition forces one to localize redox H^+ transport to site I or prior to it, which in turn implies some correlation between NAD^+ or FAD reduction and acid rate, which we have shown does not exist[18].

Although oligomycin and rutamycin were apparently unable to penetrate the tissue, the action of aurovertin in inhibiting acid rate shows that the terminal step in intramitochondrial ATP synthesis is required for this process, hence no intramitochondrial intermediates can serve as the energy source for acid secretion.

In addition the action of atractyloside in inhibiting acid secretion shows that in fact extramitochondrial ATP is required. The data therefore obtained by the use of inhibitors show that the system involved in acid transport is mitochondrial in nature, and that cytoplasmic ATP is the necessary substrate for H^+ secretion. All the data discussed are in fact consistent with this view, while several secondary assumptions would be necessary in order to explain these data either by a redox theory, or on the basis of an extramitochondrial source of energy.

Since with the use of these inhibitors, it has been possible to dissociate Cl^- transport from acid secretion, the conclusion seems warranted that the metabolic dependence of these two processes is different. The concentrations of inhibitors used would be expected to reduce mitochondrial metabolism to very low levels, and this expectation has been confirmed both by measurements of oxygen consumption and by direct observation of the components of the redox chain. Hence it is tempting to postulate the involvement of compartmental glycolytic reactions in movement of this ion, which would account for the data presented above. This would involve possibly either the glyceraldehyde-3-phosphate phosphoglycerate kinase couple or the phosphoenolpyruvate kinase enzyme. Alternatively, the residual Cl^- transport may be the function of a glycolytic system in another cell type in the mucosa, such as the surface cell[19].

Finally, we did not observe any qualitative differences between the transport responses of Necturus or frog gastric mucosa to any of the inhibitors used. In fact, the responses of this tissue, apart from the CN^- concentrations required, were similar to those expected for tissues of mammalian origin. That gastric mitochondria have similar properties to other types has already been demonstrated[20], and it would appear that the metabolism of this tissue as related to transport is also not species specific.

ACKNOWLEDGEMENTS

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