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THE ACTION OF AMYTAL ON FROG GASTRIC MUCOSA

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SUMMARY

The frog gastric mucosa has been shown to be sensitive to amytal. At 2 mM acid secretion was completely inhibited with a rise of resistance, fall in short-circuit current and no significant change in potential difference. Simultaneously there was 75 % inhibition of O_2 consumption and 50 % depression of ATP levels. Dual-beam spectrophotometric studies of intact mucosa with amytal showed a crossover point between NAD^+ and FAD. The microsomal NADH oxidase ferricyanide reductase has also been shown to be amytal sensitive. Cl^- transport was relatively insensitive to amytal, suggesting a qualitative distinction between the mechanisms underlying the transport of H^+ and Cl^- in the mucosa. This was further brought out by the effects of anoxia in which H^+ transport was inhibited at 5 min but Cl^- transport at minimally 20 min following the onset of anoxia.

INTRODUCTION

Although amytal has been shown to inhibit acid production by the stomach, using a closed-sac technique[1] its effect on other parameters, such as Cl^- transport, potential difference and resistance has not been determined. Moreover a biochemical analysis of its mode of action in this particular tissue, by O_2-consumption measurements, ATP-level determination would establish the type of inhibition occurring in the intact tissue. Of particular importance is the use of dual-beam spectroscopy to localise the action of amytal in the redox chain, to correlate changes in the steady state of redox components with changes in transport.

METHODS

All studies to be reported here were carried out on *Rana pipiens* gastric mucosa. The frogs were obtained from Lemberger and Co., Oshkosh, Wisc., and stored in running water at room temperature for several days prior to use.

Secretion studies

The gastric mucosa was mounted between two lucite chambers as previously described[2]. In this system the potential difference was measured *via* a pair of matched calomel electrodes with renewable KCl junctions, and a vacuum-tube voltmeter

Resistance was calculated from the change in potential difference obtained when a current of 10 μA was sent in either direction through the membrane. Short-circuit current was measured as the amount of current required to reduce the potential difference to zero after sending current for about 30 sec. Acid secretion was measured by the pH stat method of DURBIN AND HEINZ[3]. $^{36}Cl^-$ fluxes were determined in a Nuclear–Chicago scintillation counter, and quench correction was performed using the channels-ratio method as previously outlined[2]. The mucosa was oxygenated with 95 % O_2–5 % CO_2 and in anoxic experiments with 95 % N_2–5 % CO_2. Nutrient solutions contained 118 mM Na^+, 4 mM K^+, 0.8 mM Mg^{2+}, 1.7 mM Ca^{2+}, 18 mM HCO_3^-, 109 mM Cl^- and 10 mM glucose, whereas secretory solutions contained 105 mM Na^+, 4 mM K^+ and 109 mM Cl^-. For Cl^--free conditions Cl^- in bathing media was replaced by SO_4^{2-}. Amytal was added at varying concentrations to the nutrient solution. Fresh sodium amytal solutions were made up daily and all chemicals were reagent grade and solutions made up in glass-distilled water.

O_2-consumption studies

These experiments were performed in a chamber of a design essentially similar to the above, except that magnetic stirring was employed, and the construction material was O_2-impermeable Kel F rather than lucite. Clark O_2 electrodes were used with teflon membranes, and the output of the O_2 amplifier was fed to a scale-expansion system consisting of a bucking voltage and an Esterline Angus recorder. The mucosa was mounted as before, and solutions as above were employed, but were gassed for 30 min with 95 % O_2–5 % CO_2 prior to use. O_2 electrodes were calibrated each day in air, air-equilibrated solutions, and glucose solutions treated with glucose oxidase. At the termination of the experiment 20 mM CN^- was used to reduce the O_2 consumption to zero, to ensure that there were no undetected leaks of O_2 in the system. After an initial period of equilibration, O_2 measurements were performed for 30 min under control conditions. Then amytal was added at 2 mM concentration and O_2 consumption determined for a further 40 min. O_2 consumption was calculated directly from the deflection on the recorder and expressed as μA O_2 used per h per cm^2.

Spectrophotometric studies

These studies were performed in conjunction with Dr. W. S. REHM and Mrs. S. SAUNDERS. Frog mucosa was mounted in a lucite chamber which was designed to fit into the standard cell compartment of an Aminco Chance dual-beam spectrophotometer, and with no current sending agar electrodes, but a clear plastic window on either side of the mucosa. Acid secretion was measured by the usual pH stat method, potential difference *via* calomel electrodes with KCl bridges and circulation maintained by airlift systems. The bathing solutions and gas mixtures were as described above.

The following pairs of wavelengths were used to determine the redox state of each component measured: NAD^+, 374/340 mμ; FAD, 510/465 mμ.

The experimental procedure in each study was to allow the system to come to equilibrium under conditions of oxygenation. After both the rate of H^+ secretion and the absorbance of any one particular redox component had stabilized, anoxia was induced with 95 % N_2–5 % CO_2. The gases used in these experiments were specially prepared and calibrated. Recording of absorbance and acid rate was con-

tinued until acid rate was zero and a stable absorbance had been produced. O_2 was readmitted and acid rate and absorbance allowed to return to control conditions. At this point 2 mM amytal was added to the nutrient side and recording continued to a new steady state. This was followed by a further cycle of anoxia.

In some experiments either before or after adding amytal, uncoupling was induced with dinitrophenol, sodium arsenate or carbonyl cyanide metachlorophenylhydrazone. Following stabilization, an N_2–O_2 cycle was induced and the absorbance recorded as above.

Estimation of ATP levels

Gastric mucosae were incubated with gassing in 95 % O_2–5 % CO_2 in nutrient solutions with varying concentrations of amytal for 30 min at room temperature. The mucosa was chilled, the mucosal cells scraped off with a glass slide, homogenized, heated to 100° for 4 min, and centrifuged. The supernatant was assayed for ATP levels by the luciferin luciferase method with an arsenate buffer according to STREHLER[4]. Recovery of added ATP was better than 90 %, and no interference by ADP could be detected. Protein analysis was carried out on an aliquot of the homogenate by the method of LOWRY *et al.*[5]. Results were expressed as μmoles ATP $\times$ 10^{-2} per mg protein.

Enzyme assays

Gastric mucosal cell suspensions were prepared in ice-cold 5 mM EDTA. Homogenization was carried out in a Waring blender at maximum speed for 30 sec. The homogenate was spun at 1500 $\times$ *g* for 15 min, resuspended and recentrifuged two more times. The final pellet was resuspended in buffer (pH 8.4) to a concentration of about 1 mg protein per ml and homogenised for 20 sec in a Bronwill sonifier. In other preparations frog gastric microsomal preparations were made (*i.e.* 100000 $\times$ *g* precipitate) and ATPase activity was determined as previously detailed[6]. NADH oxidase activity was determined according to MAHLER[7] using ferricyanide as the electron acceptor and a Beckman Model DB recording spectrophotometer. Electron micrographs of the final preparations showed it to be virtually free of nuclei, cell fragments or mitochondrial contamination. Micrographs of the ultrasonic preparation from the EDTA precipitate showed a large preponderance of smooth membrane fragments. Protein analysis was again performed according to the LOWRY method[5] and units of NADH oxidase activity were expressed as Δ 0.001 *A*/min per mg protein.

Summary of methods

The action of amytal on the H^+ and Cl^- transport mechanisms of the frog gastric mucosa was measured with a simultaneous determination of the effect on O_2 consumption, ATP levels and redox systems of this tissue. The microsomal enzyme sensitivity to amytal was also measured. By these methods, the action of amytal in frog mucosa could be compared to its action in other tissue.

RESULTS

Secretion

Acid secretion was inhibited after 30 min by 2 mM amytal. During the decline in acid secretion there was a rise in potential difference and short-circuit current

initially, with little alteration in resistance. The potential difference then fell to about initial values and resistance increased. Measurements of Cl^- flux showed that only the Cl^- associated with H^+ was inhibited at this stage (Fig. 1). The residual potential difference was maintained for about 90 min after the addition of amytal at this level.

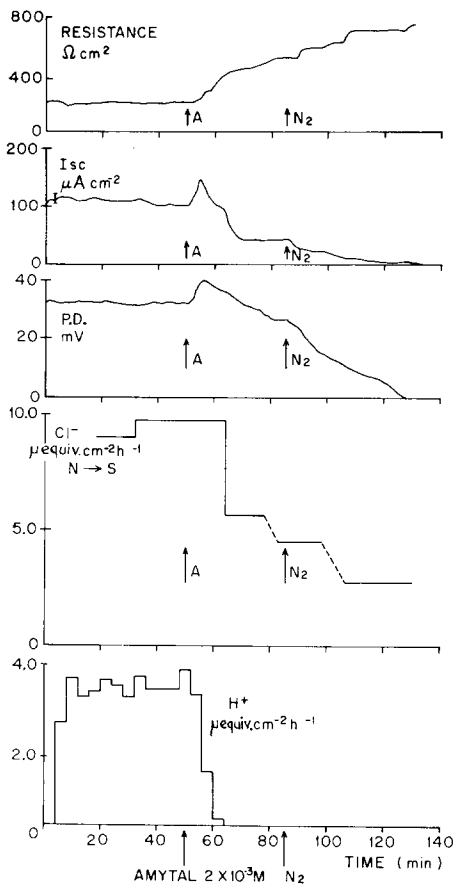

Fig. 1. The effect of 2 mM amytal (A) and anoxia on resistance, short-circuit current (I_{sc}), potential difference (P.D.), Cl^- flux (N → S) and acid rate in the frog mucosa *in vitro* in Cl^- solutions.

At 20 mM amytal, however, there was a rapid fall in Cl^- flux associated with the fall in H^+, and at 0.2 mM there was no significant effect on H^+ or Cl^- transport over 60 min. The data obtained at the 30-min period are summarized in Table I. Since the residual potential could be due to either a Cl^- diffusion or electrogenic potential or a combination of both, the effect of period of anoxia and reoxygenation was studied.

After N_2 was admitted in the amytal-treated mucosa, approx. 40 min were required for the potential difference to be reduced to zero. Upon reoxygenation there was a rise of the potential difference to a stable value after 5 min. During this time there was a rise in the resistance and a fall in net Cl^- flux with anoxia and a fall in resistance and a rise in Cl^- flux upon readmission of O_2 (Fig. 2). These results suggest a functioning Cl^- pump at this concentration of amytal (2 mM) as well as a Cl^-

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TABLE I

THE POTENTIAL DIFFERENCE, SHORT-CIRCUIT CURRENT, RESISTANCE, H^+ RATE AND Cl^- FLUX ($N \rightarrow S$, $S \rightarrow N$) AT 0 min AND AT 30 min FOLLOWING 2 mM AMYTAL

The results given are means of 20 experiments ± S.E. of the mean; ten Cl^- fluxes in either direction.

	H^+ ($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)	*Potential difference* (*mV*)	*Resistance* ($\Omega \cdot cm^2$)	*Short-circuit current* ($\mu A \cdot cm^{-2}$)	Cl^- *flux* ($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)	
					$N \rightarrow S$	$S \rightarrow N$
Control	3.44 ± 0.2	23.0 ± 2.0	141 ± 24	109 ± 8	10.1 ± 0.49	4.5 ± 0.20
25–30 min after amytal	0.2 ± 0.18	21.5 ± 1.5	331 ± 40	45 ± 7	4.7 ± 0.53	2.6 ± 0.23
Change	−3.1 ± 0.1*	−1.5 ± 1.7	+190 ± 34*	−64 ± 6*	−5.4 ± 0.58*	−1.9 ± 0.22*

* *t* test significant at 0.01 level.

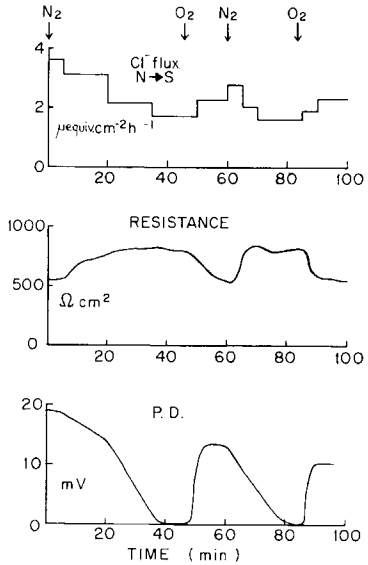
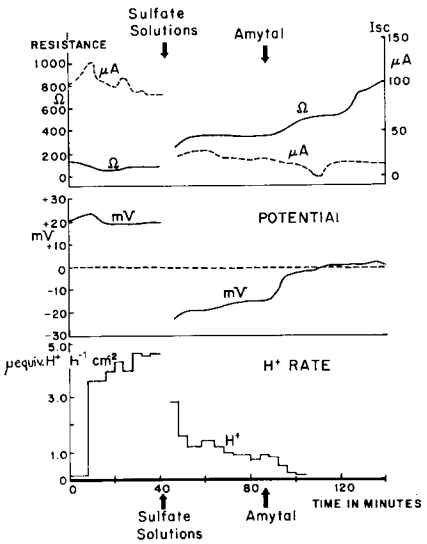


Fig. 2. The effect of cycles of anoxia on the amytal-inhibited frog mucosa in Cl^- solutions ($H^+ = 0$). P.D., potential difference.

diffusion potential which may account for a considerable fraction of the measured potential difference when H^+ rate is zero.

In mucosae bathed with a non-transported anion (SO_4^{2-}), the inverted potential difference and H^+ rate fell concomitantly to zero upon the addition of amytal (Fig. 3), as demonstrated by REHM AND LEFEVRE[8] for dinitrophenol inhibition under identical conditions.



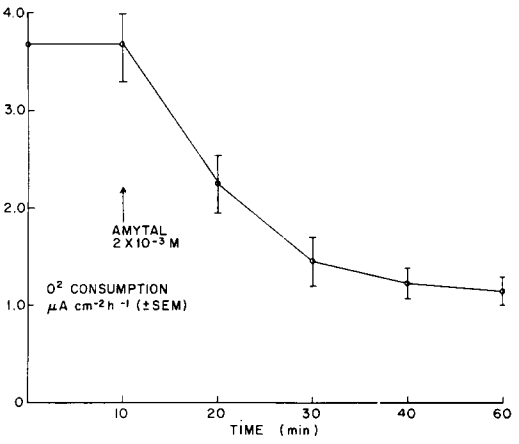


Fig. 3. The effect of 2 mM amytal on the frog mucosa in SO_4^{2-} solutions.

Fig. 4. The effect of amytal on O_2 consumption of the chambered frog mucosa (mean ± S.E. of the mean of six experiments).

O_2 consumption

Since amytal is essentially a redox inhibitor, it would be expected to strongly inhibit O_2 consumption. From Fig. 4 it can be seen that at 2 mM amytal there was about 75 % inhibition of consumption. The calculated $\Delta H^+/\Delta O$ value was 1.6, compatible with a redox theory of secretion.

ATP levels

Concomitant with reduction in electron transfer *via* NAD^+-linked dehydrogenases, the synthesis of ATP would be expected to be markedly depressed. Fig. 5 shows the effect of varying levels of amytal on the frog mucosal ATP level. The maximal drop occurred at 0.2 mM amytal which is not inhibitory to secretion at 30 min. Successive increments progressively decrease ATP levels in the mucosa and at the concentration studied in this paper, namely 2 mM, there was about 50 % residual ATP.

Spectrophotometric studies

In the chambered mucosa, changes in state of NAD^+ (ΔA at 374/340 mμ) were recorded during cycles of deoxygenation and oxygenation. There was a marked

increase in the absorbance with N_2, with an overshoot before the final level was reached with a zero H^+ rate after 5 min (Fig. 6). Upon readmission of O_2 there was a decrease in the ΔA, showing oxidation of NADH. Secretion started after about 3 min following the readmission of O_2 in this type of experiment. After the H^+ rate had stabilized, addition of 2 mM amytal resulted in a slow reduction of NAD^+, which

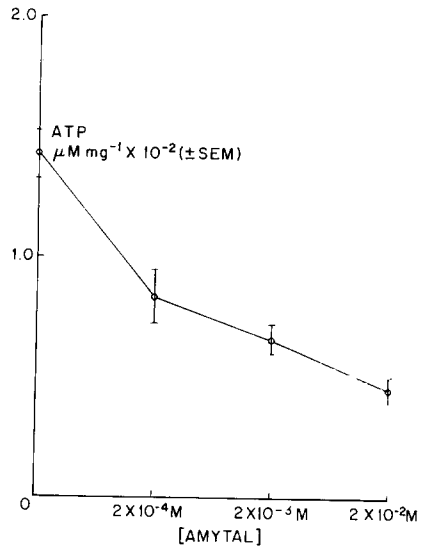


Fig. 5. The effect of amytal on ATP levels in frog gastric mucosa (six observations at each amytal level ± S.E. of the mean).

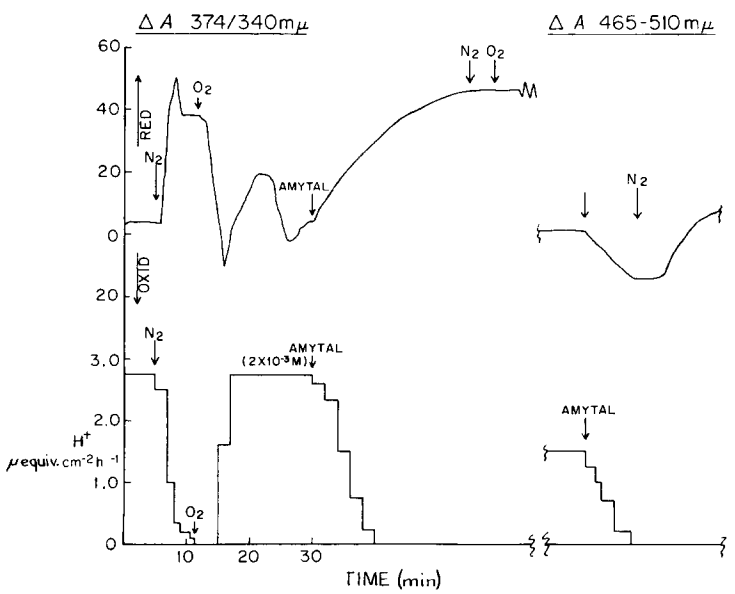


Fig. 6. The effect of anoxia and amytal on the ΔA at 374/340 mμ (NAD^+) and ΔA at 465/510 mμ (FAD) of the frog gastric mucosa in the Aminco Chance dual-beam spectrophotometer.

had not reached a maximum when H^+ was zero. Admission of N_2 followed by O_2 did not alter the observed absorbance. Although the action of amytal was reversible, experiments on the other redox enzymes were usually performed in separate experiments. The state of FAD in the mucosa was determined by ΔA at 510/465 mμ. When the tissue was made anoxic, reduction (*i.e.* decrease in ΔA) occurred as expected, and when O_2 was readmitted there was reoxidation with little evidence of an overshoot. When acid secretion was re-established, 2 mM amytal was added with ensuing oxidation of FAD (Fig. 6). If the tissue was then made anoxic, a slow reduction of FAD was observed in keeping with the reduced rate of electron transfer from NADH.

In experiments where arsenate and the other uncouplers were added following amytal, further oxidation of FAD resulted (Fig. 7). When arsenate was added prior to addition of amytal there was oxidation of FAD and further oxidation with amytal. Similar results were obtained with the other uncouplers studied.

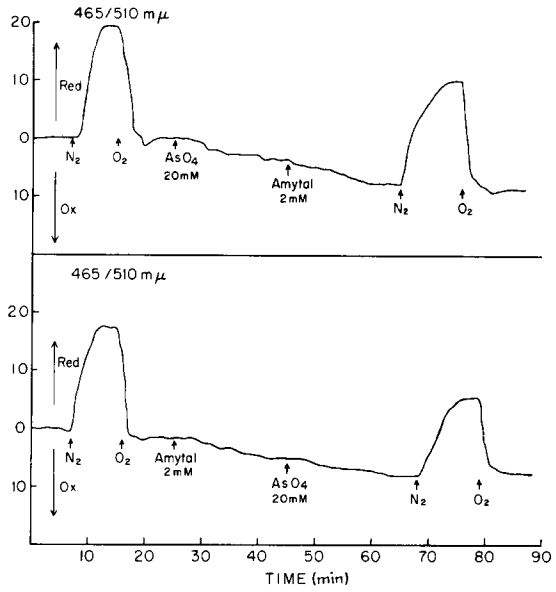


Fig. 7. The effect of sodium arsenate and amytal on the ΔA at 465/510 mμ (FAD) of the frog gastric mucosa. In the top figure arsenate was added before amytal, while the reverse sequence was used in the lower figure.

Enzyme studies

Since the mitochondrial system is not unique in catalysing NADH oxidation, a membrane preparation was studied with respect to the effect of amytal on the NADH oxidase system. In this preparation 2 mM amytal had no detectable effect on the activity, but at 6 mM there was approx. 30 % inhibition. There was also a lag in the onset of inhibition which was not found when amytal was premixed with the enzyme. This membrane preparation also exhibited ATPase activity, which was sensitive to SCN^- (a potent secretory inhibitor) but not to amytal. The NADH oxidase activity, however, was found to be SCN^- insensitive (Table II).

TABLE II

THE EFFECT OF AMYTAL AND SCN^- ON FROG GASTRIC MICROSOMAL NADH OXIDASE AND ATPASE

The results given are means of five experiments ± S.E. of the mean.

	NADH oxidase (Δ 0.001 $A \cdot min^{-1} \cdot mg^{-1}$)	*ATPase* ($\mu moles\ P_i \cdot min^{-1} \cdot mg^{-1}$)
Control	318 ± 31	1.71 ± 0.12
SCN^- (1 mM)	310 ± 26	0.46 ± 0.12
Amytal	216 ± 15	1.73 ± 0.29

Summary of results

Amytal at 2 mM reduced the H^+ secretory rate to zero, with lesser effect on net 'non-acidic' Cl^- flux. At this amytal concentration there was a 50 % depression in ATP levels, a 75 % inhibition of O_2 consumption, with a reduction of NAD^+ and oxidation of FAD. At 6 mM amytal the NADH oxidase system present in the microsomal fraction was 30 % inhibited, but no effect on the ATPase was found.

DISCUSSION

The action of amytal in blocking electron transport between NAD^+ and FAD in mitochondrial systems is well established. That this effect occurs in an intact transporting tissue, such as gastric mucosa, and that it can apparently account for selective inhibition of transport of one ion and not another, has not previously been reported. It appears, that in order to obtain a given effect, the concentration of amytal is critical. At 0.2 mM, little effect was noted, and at 20 mM both Cl^- and H^+ transport were inhibited. At 2 mM, however, selective H^+ secretory inhibition occurs, with an initial rise in the potential difference and short-circuit current. This latter effect is presumably the consequence of biochemical coupling between the H^+ rate and the Cl^- mechanism.

Subsequently the potential difference was well maintained, but after about 60 min there was a steady decline in the potential difference with a rise in the resistance. The measurement of Cl^- flux established that at 30 min following amytal the net Cl^- transport was unchanged, apart from the fraction accompanying H^+.

In SO_4^{2-} solutions, amytal reduced both H^+ secretion and potential difference to zero, since SO_4^{2-} is not transported by *Rana pipiens* gastric mucosa[9].

The maintained potential difference in Cl^- solutions, in the absence of an acid rate with 2 mM amytal could be the result of a residual diffusion potential (cell to lumen) established prior to the amytal inhibition. Anoxia for 40 min resulted in the fall of potential difference to zero, and reoxygenation re-established 60 % of the potential difference within 5 min. This implies that the mechanism for establishing the potential difference is still operative 70 min following amytal addition.

The biochemical changes in the tissue must thus account for the changes in H^+ transport with sparing of the Cl^- mechanism, and might yield some insight into the biochemical dependence of this transport.

Dual-beam studies showed that there was a change in the steady state of NAD^+ towards reduction, and of FAD towards oxidation. The reduction of NAD^+, however, is incomplete at the time that the H^+ rate is zero, whereas with anoxia

there appeared to be simultaneity of inhibition of H^+ and NAD^+ reduction. It can be concluded, however, on the basis of the amytal effect, that there is no simple correlation between NAD^+ metabolism and the H^+ rate.

Since the majority of cellular oxidations traverse the NAD^+ pathway, 70 % inhibition of O_2 consumption was observed, coupled with a 50 % depression of ATP levels. The measurement of these two parameters appears critical for an evaluation of either a redox or an ATPase theory of secretion. The results obtained here raise difficulties for either theory. The overall $\Delta H^+/\Delta O$ ratio found of 1.6, although compatible with a simple redox theory, is undoubtedly an underestimate since it is highly unlikely that the NAD^+-dependent O_2 consumption is entirely concerned with H^+ production. The ratios previously found depend on the technique used[10]. Since there was 50 % residual ATP, an explanation of the action of amytal based on ATP depletion requires some second-order assumptions, the most obvious one being compartmentation of ATP, with depletion of that fraction of the ATP concerned with H^+ transport, or inhibition of the transport system by ADP. Another alternative, namely that amytal has a direct action on the cell membrane, is not excluded by our data, since although the microsomal ATPase was unaffected by amytal, the NADH oxidase was inhibited by 30 %, albeit at a higher concentration than 2 mM. The function of this system is not well understood, but appears to be associated with microsomes, rather than plasma membrane.

Thus, using currently available biochemical techniques, the action of amytal on the gastric mucosa has been shown to consist of inhibition of oxidation of NADH with concomitant reduction of O_2 consumption and ATP levels. As a consequence of these actions, there is inhibition of the H^+ rate, with little effect on Cl^- transport. Anoxia has also been shown to dissociate H^+ and Cl^- secretion. It is clear therefore that these two transport systems are clearly differentiated in their metabolic dependence. On the assumption that ATP is the energy source for H^+ and Cl^- secretion, one simple explanation is that H^+ is dependent on mitochondrially generated ATP, whereas Cl^- transport may utilise glycolytic ATP under certain experimental conditions.

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