J. Membrane Biol. 35, 189–204 (1977)

Redox Involvement in Acid Secretion in the Amphibian Gastric Mucosa

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Received 17 September 1976; revised 18 October 1976

Summary. Gastric fundic metabolism was studied by spectroscopic observation in frog mucosa during transitions of secretory status in vitro and by direct measurement of pyridine nucleotides and associated metabolites in biopsies of dog fundic mucosa also during secretory transition. In frog, inhibition of spontaneous secretion by an H_2 antagonist resulted in oxidation of the redox components from flavin adenine dinucleotide (FAD) to cytochrome a_3. Addition of histamine resulted in reduction of these components with onset of secretion by about 50%. In contrast, the effect of apparently, burimamide and subsequently histamine on the ratio of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide, reduced (NAD^+/NADH) was relatively slight. Further, the presence of burimamide substantially reduces the effect of amytal on the pyridine nucleotide spectrum and abolishes the effect of amytal on FAD and the cytochromes. Measurements of lactate, pyruvate, α-ketoglutarate, NH_3 and glutamate in the dog showed that whereas the calculated NAD^+/NADH ratio in the cytoplasm declined with onset of secretion, the calculated mitochondrial ratio rose. No change was noted in the nicotinamide adenine dinucleotide phosphate/nicotinamide adenine dinucleotide phosphate, reduced ($NADP^+$/NADPH) ratio. It is concluded that (1) H_2 antagonists act by blocking substrate flow into the mitochondrial respiratory chain, (2) conversely, histamine stimulation acts at the level of substrate mobilization, and (3) there may be a cross-over in the mitochondrial chain between NAD^+ and FAD.

The gastric mucosa is capable of producing a H^+ gradient of one millionfold magnitude as measured by the blood to lumen pH difference. The nature of the primary energy source for this acid secretion, despite much experimentation, remains controversial. Historically, this controversy has settled on two mechanisms [3, 9, 14, 16, 17]. The first mechanism suggests a redox carrier which accepts reducing equivalents from mitochondria presumably supplied by a generalized substrate mobilization and shuttles these to the parietal cell membrane followed by oxidation and secretion of H^+ into the gastric lumen. Presently, it is the absence of information concerning this carrier and the redox reaction in the membrane and of the electron carrier from membrane to mitochondria which limits the development of the 'redox hypothesis'. The second

mechanism proposes that a tissue-specific ATPase provides the pump necessary to move protons across the parietal cell membrane into the gastric lumen.

The strongest argument favoring a direct role for ATP in acid secretion is provided by work with subcellular fractions of the fundic mucosa [5, 15].

Resolution of the problem of the specific participation of the redox and ATPase systems in secretion in the intact mucosa can be approached by two methods which allow measurements of metabolites during secretion. In the first, Lowry enzyme cycling methods may be used to fluorimetrically measure metabolite levels of the parietal cell areas both preceeding and after the establishment of steady-state acid secretion [13, 16]. This technique allows measurement of many metabolites but not FAD or the cytochromes. In the second, the split-beam spectrophotometer with its ability to accommodate samples with large light-scattering properties can be used to monitor cytochrome and pyridine nucleotide levels during changes in the secretory rate of the intact gastric mucosa of frogs [8, 10, 16].

It is the purpose of this paper to compare results with the split-beam spectrophotometer and the cycling methods to characterize the complex redox changes which result from the transition between burimamide-inhibited or resting and histamine-stimulated secretion. This characterization yields information in two ways. First, it allows a comparison to be made between secretion-induced steady-state changes and those of classical mitochondrial activity states. This in turn could indicate the limitation of a specific component vital to the secretory process. Secondly, the existence of a cross-over point induced by the inhibition or stimulation of secretion would indicate the specific locus of interaction of that process with the respiratory system. This identification may be a necessary initial step in the demonstration that the observed changes in mitochondrial electron transport activity induced by the inhibition or stimulation of acid secretion result from the specific involvement of a redox reaction with the secretory mechanism.

Materials and Methods

Spectrophotometric Studies

Bullfrogs (*Rana catesbeiana*) were obtained from the Jacques Wiel Company. Prior to use they were kept in tanks supplied with running tap water and fed daily with crickets. Before each experiment the frogs were decapitated, pithed and the stomachs perfused

with three 20-ml injections of nutrient solution into the dorsal ascending aorta to remove hemoglobin. The stomach was then removed and placed in an aerated nutrient solution where the mucosa was removed by blunt dissection. This mucosa was then stretched on a cork ring and placed in a double segment Plexiglas chamber designed to fit into the Aminco DW-2 spectrophotometer allowing for a control and experimental side. To hold the two parts of the stomach motionless within the Plexiglas chamber, the tissue was pressed secretory surface down against a stainless steel mesh. Inside the chamber, the tissue was bathed with solutions from a four-chambered reservoir. The two serosal reservoirs contained 15 ml of a solution containing (mM): 91 Na^+, 5 K^+, 1.5 Mg^{++}, 1.0 Ca^{++}, 20 HCO_3^-, 1.0 $H_2PO_4^-$, 5.0 glucose and 80 Cl^- at pH 7.4. The mucosal reservoirs held 10 ml of a solution containing (mM): 70 Na^+, 5.0 K^+, 1.5 Mg^{++}, 1.0 Ca^{++} and 80 Cl^-, initially at pH 5.5.

Oxygen tension was maintained by bubbling both mucosal and serosal reservoirs with a mixture of 95–5% O_2/CO_2 while utilizing a 250-ml air trap to avoid mixing solutions through the mucosal or serosal tubes. Uniform flow rate through the optical chambers was maintained by a peristaltic pump connected between the reservoirs and the optical chamber.

For the secreting mucosa, pH was maintained at 4.7 by a Radiometer pH-stat assembly consisting of a PHM 64 Research pH meter, a TTT 60 Titrator, REC 61 Servorecorder equipped with an REA 160 Titrigraph Module and an ABU 12 Autoburette.

Burimamide was supplied by Smith Kline and French, Amytal by Eli Lilly and the remaining reagents by Sigma. Solutions were stored at 4 °C and prepared fresh weekly.

Absorption measurements were made in the Aminco DW-2 Splitbeam Spectrophotometer. The absorption ranges, usually either 0.1 or 0.2 O.D. full scale were calibrated before each experiment. The wavelength accuracy was calibrated by comparison of the instrument settings to the 550 nm absorption peak of reduced cytochrome *c*. The spectral bandwidth was set at 15 nm for each experiment.

The concentrations of the absorbing species were calculated according to the Beer Lambert law: $A = \varepsilon \times b \times c$.

$A =$ absorbance as measured in optical density units ($\log I/I_0$)

$b =$ optical path length (assumed to be the thickness of the tissue, 0.5 mm)

$\varepsilon =$ Molar absorptivity ($cm^{-1} \times mM^{-1}$)

$c =$ concentration (mM).

ε values were those calculated by Chance and Williams [2].

Component	Wavelength	*E*
NADH	340–374 nm	6
FADH I	465–510 nm	11
Cytochrome b_a	564–575 nm	20
Cytochrome $c(c_1)$	540–550 nm	19
Cytochrome a_a	605–630 nm	16
Cytochrome a_3	445–455 nm	60

Absorption changes were monitored continuously after each experimental procedure, but the changes at 30 min were used for the calculations, at which time steady state had been invariably established.

Measurements of Metabolites by Cycling Methods

The experimental details involved in obtaining testing and secreting biopsy samples from anesthetized dogs have been detailed in a previous publication [18]. Briefly, the fasted dog was anesthetized, the abdomen opened, the stomach opened, and several biopsies taken. These were denoted as resting samples by the absence of measurable acid secretion into a Lashley cup. Histamine was then infused and samples taken at reddening of the mucosa and then with onset of acid secretion. Secretion was quantitated by titration of the fluid in the Lashley cup, and ranged from 100–300 μmoles $H^+cm^{-2}hr^{-1}$.

The biopsy was immediately frozen in liquid N_2 and sectioned at −20 °C. Parietal cell regions were defined by staining for succinic dehydrogenase in every fourth section; the other sections were lyophilized and the metabolites measured under oil wells. The methods used for measurement of the pyridine nucleotides and lactate and pyruvate have been published previously [18].

The principle of the fluorimetric cycling procedure is to convert a metabolite quantitatively to oxidized or reduced pyridine nucleotide, destroying the unwanted form with acid or alkali. The remaining pyridine nucleotide is then measured by a cycling technique, for example, using alcohol, oxaloacetate, alcohol dehydrogenase and malic dehydrogenase. One of the products of the cycling reaction (e.g. malate in the example) is now measured by coupling to the formation of reduced pyridine nucleotide and direct fluorimetric measurement of the latter. In this way levels of about $10^{-12}-10^{-13}$ moles of metabolite are converted to 10^{-9} moles of pyridine nucleotide for the final step [13].

To calculate the ratio of free oxidized and reduced pyridine nucleotide in cytosol and in mitochondria, pairs of metabolites are used on the assumption that firstly, the enzyme interacting with the metabolites is present only in the compartment of interest, and secondly, that it has sufficient activity to maintain reactant equilibrium. For cytoplasmic calculations, the reactions catalyzed by lactic dehydrogenase or α-glycerolphosphate dehydrogenase may be used and give similar data in the stomach. For the mitochondrial compartment the reaction catalyzed by β-hydroxybutyrate dehydrogenase or glutamic dehydrogenase may be used. Measured levels of β-hydroxybutyrate dehydrogenase were sufficiently low so that one could not be sure that adequate quantities of this enzyme were present, hence the levels of α-ketoglutarate, NH_3 and glutamate were used for calculation of the ratios of NAD^+ to NADH in mitochondria. These methods therefore have the advantage of being able to discriminate between changes in NAD^+ and $NADP^+$, as well as discriminate between changes in different cellular compartments and cell types (e.g. mucus as opposed to parietal cells by serial sectioning). Hence, they are complementary to the spectroscopic techniques but being steady-state measurements require a much larger secretory transition than is obtained in the frog, hence necessitating the use of in vivo dog tissue.

α-Ketoglutarate. The sample was treated under oil in specially constructed oil wells with 1 μliter 0.05 N NaOH for 20 min at 80° C. After cooling to room temperature, 2 μliters of a solution containing 200 mM imidazole acetate buffer, pH 6.6, 20 μg/ml glutamate dehydrogenase, 2 μM NADH, 50 mM NH_4OAc, 200 mMADP and 0.05% BSA were added and incubation continued for 30 min. Residual NADH was then destroyed by addition of 1 μliter 0.5 N HCl and incubating for 30 min. One μliter of this reaction mixture was then added to 100 μliters of a solution containing 294 μg/ml alcohol dehydrogenase and 45 μg/ml malic dehydrogenase, with in addition 100 mM Tris HCl buffer, pH 8.0, 300 mM ethanol, 2 mM mercaptoethanol, 0.02% BSA and 2 mM oxaloacetate. After cycling for 60 min at 28 °C, the reaction was stopped by heating to 100 °C for 10 min and 7 μliters were added to 1 ml of indicator reagent.

The latter contained 50 mM 2-amino-2-ethyl propanol buffer, pH 9.9, 0.02 mM NAD^+, 10 mM glutamate, 5 μg/ml malic dehydrogenase, 2 μg/ml glutamate-oxaloacetate transaminase. After 10 min at 22° C, the NADH produced from the oxidation of the malate produced by cycling was measured fluorimetrically [12].

Glutamate. The lyophilized sample was treated with, under oil, 1 μliter of 0.02 N HCl for 20 min at 80 °C to destroy any NADH present. After cooling to room temperature, 2 μliters of a reagent containing 50 mM Tris-acetate, pH 8.4, 50 μg/ml of glutamic dehydrogenase, 100 μM NAD^+, 100 μM ADP and 0.02% BSA was added and the reaction allowed to proceed for 30 min at room temperature. To this 1 μliter of 0.25 M NaOH was added to destroy excess NAD^+ and the mixture heated at 80° C for 20 min. One μliter of this mixture was then added to 100 μliters of cycling reagent as above but containing 76 μg/ml of alcohol dehydrogenase and 11 μg/ml of malic dehydrogenase. Cycling was carried out as above and the indicator step was also the same [12].

NH_3. Since lyophilization would remove NH_3, this metabolite was measured in perchloric acid extracts of the frozen biopsies. The frozen sample was pulverized at −20 °C and 100 μliters of 0.1 N HCl in 99% methanol added. The sample was dispersed and 1 ml of 0.3 M perchloric acid with 1 mM EDTA added in ice. The mixture was centrifuged and the supernatant neutralized by 200 μliters of 1.6 N KOH, 0.4 M imidazole. After a second centrifugation the supernatant was stored at −80 °C [4].

For NH_3 assay 1 ml of a reagent containing 40 mM Tris-Cl, pH 8.4, 5 mM α-ketoglutarate, 0.01% BSA, 0.1 mM ADP, 200 μg/ml glutamic dehydrogenase in glycerol and 27.5 μM NADH was added to a quartz tube and the initial fluorescence measured. Then 50 μliters of the above extract were added and the reading taken after 10 min. The difference was used for NH_3 calculations.

Results

Burimamide Inhibition of Spontaneously Secreting Tissue

Since both the secretory rate and the redox changes produced in the transition from spontaneously secreting to secretagogue-stimulated steady states are very small, the spontaneous secretory rate must initially be reduced to low levels so that a stimulation in secretion will produce a measurable redox change. Burimamide, an H-2 blocker acting at the level of stimulus was added for this purpose [1].

The addition of 3 mM burimamide to the nutrient side of spontaneously secreting tissue reduced secretion from 2.5 μequiv/cm^2/hr to 0 ($n=11$) in a period of approximately 15 min. Thirty minutes after the addition of burimamide during a period of complete inhibition of secretion, a tracing was made between 320 and 640 nm of the absorption changes produced. It should be noted that burimamide had no effect on the absorption of spontaneously resting tissue ($n=5$).

The absorption profile shown in Fig. 1 indicates oxidative shifts in the absorption regions identifying FAD, cytochrome *b*, $c(c_1)$, *a* and a_3. The cytochrome $c(c_1)$ component appeared as a rounded peak with an absorption maximum at 555 nm instead of 550 nm. Cytochrome *b* absorbance was represented by a slight shoulder on the longer wavelength side of the same peak. There appeared to be oxidative changes involving NADH at 340–375 nm. However, since the changes were in general within the noise variation in this area the measurements are omitted.

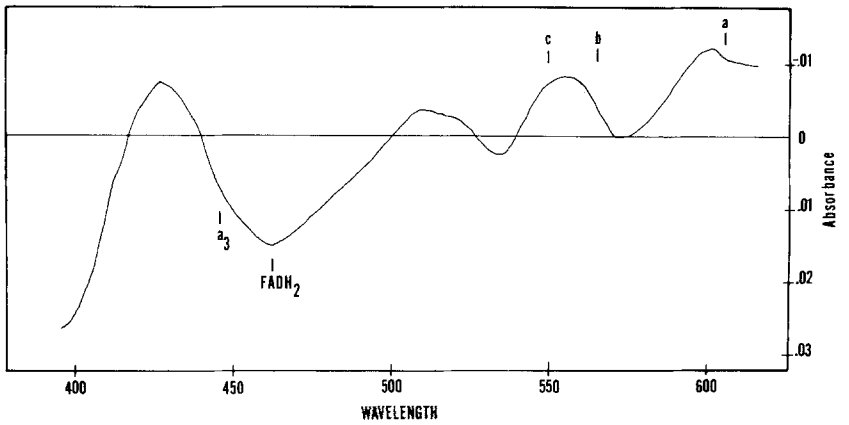

Fig. 1. Absorption spectrum induced by burimamide inhibition of spontaneously secreting frog mucosa: Steady-state absorption changes recorded 30 min after the addition of 3 mM burimamide to the nutrient side of spontaneously secreting frog mucosa. The baseline was drawn with both sides of the mucosa spontaneously secreting. Note that oxidation is upward

The reference wavelengths generally used in mitochondrial studies are a reasonable fit for the baseline drawn through a 575 nm isosbestic point [2]. Using the extinction coefficients measured by Chance, the absorbance changes from five experiments have been converted to concentration changes and recorded in Table 1, row 1. Since uncertainty is introduced into the calculation with the measurement of the pathlength due to light scattering within the tissue and the resolution of unique cytochromes at the measured reference and absorption pairs, these concentrations are only estimates of the true tissue concentration (cf. actual measurements in the dog).

The average of the concentration changes of reduced component relative to cytochrome *a*, shown in Table 1 is: cytochrome *b*, 1.2; cytochrome $c(c_1)$, 1.2; cytochrome *a*, 1.0; cytochrome a_3, 0.7. The variation from unity of this average change found in cytochromes *b* and $c(c_1)$ relative to cytochrome *a* is not experimentally significant due to the large variation between experiments. The ratio of the FAD change relative to cytochrome *a* is 4.3:1. The average values for the oxidative changes induced by the burimamide inhibition of secretion is: FAD -0.017 mM, cytochrome *b* -0.005 mM, cytochrome $c(c_1)-0.005$ mM, cytochrome *a* -0.004 mM, and cytochrome $a_3-0.003$ mM. These concentration changes represent approximately 15% of the total tissue concentration of FAD, cytochromes *b*, $c(c_1)$ and *a* as estimated below. Cytochrome a_3 was slightly

Table 1. Calculated steady-state changes produced by treatment of gastric mucosa (the values given are in mmoles/kg wet weight)

Condition	H^+ (μEquiv cm^{-2} hr^{-1})	Pyridine nucleotide	FAD	cyto *b*	cyto $c(c_1)$	cyto *a*	cyto a_3
Spontaneous to burimamide		–	-0.017 ± 0.005 ($n=5$)	-0.005 ± 0.001 ($n=5$)	-0.005 ± 0.001 ($n=5$)	-0.004 ± 0.001 ($n=5$)	-0.003 ± 0.001 ($n=5$)
Ratio			4.3	1.2	1.2	1.0	0.7
% Total	−2.5	–	15	20	15	14.2	8.8
Burimamide to histamine+ burimamide	+3.5	0.052 ± 0.004 ($n=4$)	0.055 ± 0.018 ($n=5$)	$0.012 + 0.004$ ($n=5$)	0.018 ± 0.005 ($n=5$)	0.013 ± 0.005 ($n=5$)	0.012 ± 0.004 ($n=5$)
Ratio		0.4	4.3	1.0	1.4	1.0	1.0
% Total		3.6	49	54	55	46	42
Burimamide to burimamide + N_2	0	0.141 ± 0.033 ($n=3$)	0.113 ± 0.074 ($n=3$)	0.021 ± 0.002 ($n=3$)	0.029 ± 0.003 ($n=3$)	0.025 ± 0.004 ($n=3$)	0.029 ± 0.004 ($n=3$)
Ratio		5.6	4.5	0.8	1.2	1.0	1.2
% Total		100	100	100	100	100	100
Amytal to N_2 + amytal	0	0.122 ± 0.020 ($n=3$)	0.112 ± 0.042 ($n=3$)	0.025 ± 0.011 ($n=3$)	0.034 ± 0.013 ($n=3$)	0.031 ± 0.010 ($n=3$)	0.030 ± 0.007 ($n=3$)
Ratio		4.0	3.8	0.8	1.1	1.0	1.0
% Total		100	100	100	100	100	100

– indicates oxidation.
Ratio indicates ratio of component change to cytochrome *a*.
n is number of experiments ± SEM.

less oxidized with a change accounting for 9% of its total tissue concentration.

From these observations, inhibition of acid secretion at the level of the stimulus apparently interrupts flow of reducing equivalents into the mitochondrial respiratory chain. This effect is also dependent on the presence of secretion since resting tissue does not show any redox changes following burimamide. The relative changes of the redox components also suggest, with the exception of pyridine nucleotides, relatively uniform changes and certainly the absence of a cross-over below FAD.

Histamine Stimulation of Burimamide-Inhibited Tissue

In order to record the redox shifts produced by the transition from a nonsecreting to a secreting steady state, 3 mM histamine was added to the nutrient side of burimamide-inhibited tissue. The addition stimulated secretion from 0 to 3.5 μequiv/cm^2/hr ($n=11$) in approximately 15 min. Thirty minutes after the addition of histamine, during maximal secretion, the absorbance changes were recorded from 300 to 640 nm. The tracing recorded in Fig. 2 indicates a general reduction of all respiratory chain components with the possible exception of pyridine nucleotide.

Cytochrome *a* was a peak measured at 605–630 nm. Cytochrome *b* appears as a shoulder at 564 nm on the absorption peak due to cytochrome *c*. Maximal absorption of cytochrome *c* was recorded at 555 nm. The reason for this shift has not been determined, though contributing factors may be either absorption of c_1 at 554 nm or traces of deoxyhemoglobin not removed by perfusion. The FAD peak occurs at 465 nm. NADH absorption when present appears as a broad peak from 340 to 355 nm.

The concentration changes responsible for these absorbances in five experiments were calculated and recorded in Table 1, row 2. Though there is apparent variation in the concentration of like components between experiments, the average relative change of five experiments for cytochromes *b*, $c(c_1)$, *a* and a_3 is consistently unity. The relative concentration change of FAD is higher, actually about 4.

When compared to the total concentration of tissue components, the histamine-induced reduction accounts for almost 50% of the total tissue concentration of FAD, cytochromes *b*, $c(c_1)$ and *a*. Cytochrome a_3 occupies a slightly more oxidized position with reduction accounting for 42% of its total tissue concentration.

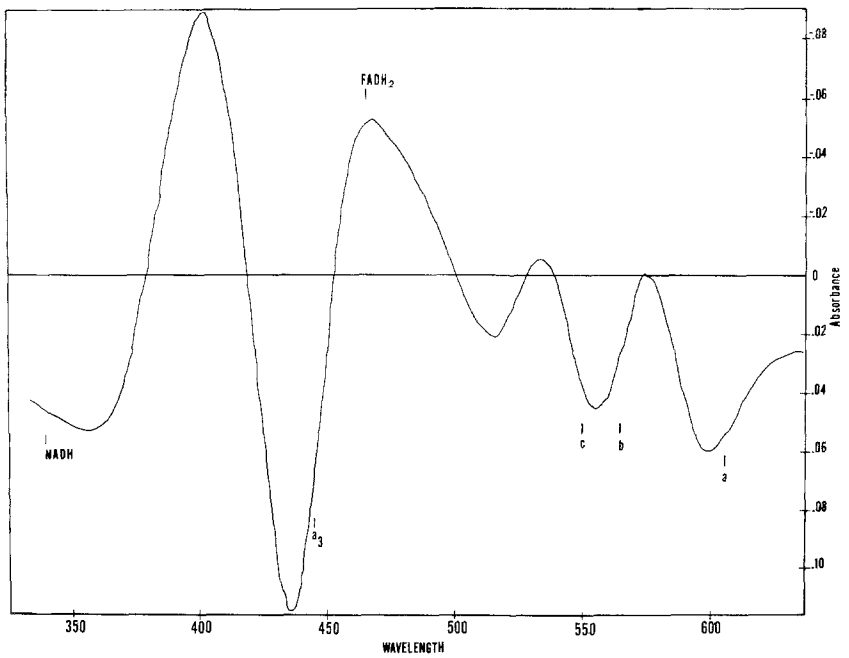

Fig. 2. Absorption spectrum induced by histamine stimulation of burimamide-inhibited frog mucosa: Steady-state absorption changes observed 30 min after the addition of 3 mM histamine to the nutrient side of frog mucosa which had been previously inhibited by the addition of 3 mM burimamide. The baseline was drawn with both sides of the mucosa inhibited with burimamide. Note that oxidation is upward

NADH was more difficult to measure due to a tendency for the 374 nm reference wavelength to shift in some experiments. Unless the 374 nm wavelength was stable, the measurements were omitted in the concentrations recorded in Table 1 (one of five experiments). Measurements indicate that NADH is much less reduced than the other tissue components. Its relative reductive change compared to cytochrome *a* is 0.4. This corresponds to only 3.6% of the total tissue NAD concentration.

These data are similar to those previously published by Hersey and his collaborators [8] in that generalized changes were observed either with stimulation or inhibition of secretion. These are in contrast, however, to reports suggesting only specific changes in cytochrome *c* [11]. A difference between the techniques is the use of CO in the latter experiments. We used 10% CO and in our hands the presence of this gas in the gassing solution did not prevent the general changes observed, hence this gas was not used routinely.

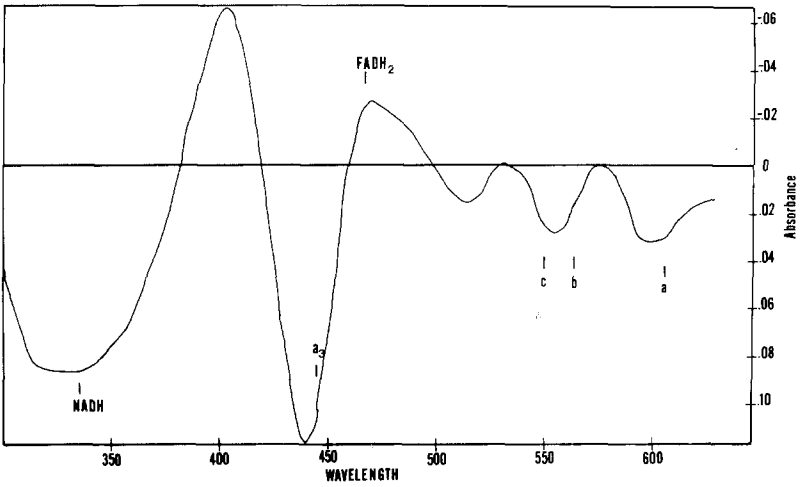

Fig. 3. N_2 reduction of amytal-inhibited tissue: Steady-state absorption changes recorded 20 min after the substitution of N_2 for the 95–5% O_2/CO_2 normally supplied the tissue in amytal-inhibited mucosa. The baseline was drawn with amytal inhibited tissue. Note that oxidation is upward

Interactions of Anoxia and Amytal

If the interpretation of the reciprocal effects of burimamide and histamine are correct, namely that it is substrate mobilization that is affected by stimulus and antagonist, then the presence of antagonist should significantly affect the redox response to amytal.

Fig. 3 shows the effect of N_2 on amytal-inhibited tissue. Since amytal, by blocking electron flow between NAD^+ and FAD results in complete oxidation of all the redox components below NAD^+ and anoxia results in complete reduction, this experiment allows calculation of total tissue concentrations of FAD and the cytochromes.

These data are given in the third row of Table 1. As can be seen there is a characteristic 1:1 shift of the cytochromes relative to cytochrome *a* and a fourfold relative reduction of FAD. Although one would anticipate that mitochondrial pyridine nucleotide is fully reduced, anoxia resulted in a considerable reduction of pyridine nucleotide, although slightly less than anoxic effect on burimamide-inhibited tissue.

This may be interpreted as being due to reduction of extramitochondrial nucleotide. This would put the ratio of extramitochondrial pyridine nucleotide to cytochromes at about 4 and account for the majority of pyridine nucleotide in the tissue.

On the hypothesis that burimamide results in complete oxidation of the mitochondrial respiratory chain, then the effect of N_2 on burimamide-treated tissue should be quantitatively identical (except for pyridine nucleotide). The fourth row of Table 1 shows that this is essentially true. Accordingly, the spectral change in pyridine nucleotide can be taken as the shift also from totally oxidized to totally reduced. As mentioned above, this shift is larger than the shift with amytal and anoxia. If amytal results in complete reduction of intramitochondrial pyridine nucleotide, then the difference in reduction between burimamide$+N_2$ and amytal$+N_2$ is due to reduction of oxidized intramitochondrial nucleotide by anoxia. This suggests that mitochondrial nucleotide comprises about 15% of total tissue nucleotide.

Accordingly, the addition of amytal to a burimamide-inhibited tissue should result in a shift of completely oxidized intramitochondrial nucleotide to completely reduced. This corresponds to a change equivalent to 17% of the total nucleotide, a reasonable agreement to the 15% deduced above. No change was observed in the subsequent members of the respiratory chain showing also that there was no electron flow into this portion of the chain with burimamide.

This then raises the question as to why only 3.6% of total nucleotide changed with histamine stimulation of secretion and whether this was due to a change in cytoplasmic or mitochondrial components. This was partially resolved by data derived from studies on dog mucosa.

Levels of Metabolites in Dog Mucosa. The data obtained are shown in Table 2. It can be seen that in terms of total pyridine nucleotide, the change in NADPH is insignificant and can be neglected in predicting the changes likely to be observed spectroscopically. In data published elsewhere [18] the NAD^+ levels in mucous cells were comparable to those of parietal cells, but the NADH levels were only 15% of those in parietal cells. With secretion the latter value actually fell, hence one may also neglect changes in mucous cells as contributing to the data. Thus there is a decline in the measured NAD^+/NADH ratio with onset of secretion in the dog corresponding to an increase of 0.228 mmoles of NADH of a total pyridine nucleotide of 2.265 mmoles. This represents a change of 10% of the total. If this were intramitochondrial nucleotide this would correspond to close to 60% reduction of that compartment based on the considerations discussed in the spectroscopic data. It is therefore essential to calculate the compartmental distribution of the change.

When this is done, it can be seen that there is a 28% fall in the

Table 2. Metabolite levels in dog parietal cell region

	Rest (mmoles/kg dry weight)	Secretion
Lactate	3.41 ± 0.46 ($n=4$)	5.93 ± 1.47[a] ($n=4$)
Pyruvate	0.424 ± 0.046 ($n=7$)	0.604 ± 0.038[b] ($n=7$)
$NAD^+/NADH$ (cytosol)	1.3×10^3	0.94×10^3
Glutamate	12.13 ± 2.04 ($n=7$)	9.34 ± 2.38 ($n=7$)
α-ketoglutarate	0.359 ± 0.37 ($n=7$)	0.404 ± 0.41 ($n=6$)
NH_3	1.92 ± 0.06 ($n=10$)	2.21 ± 0.07[a] ($n=10$)
$NAD^+/NADH$ (mitochondria)	2.94	5.0
NAD^+ (total)	1.97 ± 0.07 ($n=17$)	1.88 ± 0.07 ($n=11$)
NADH (total)	0.295 ± 0.020 ($n=17$)	0.523 ± 0.074[b] ($n=11$)
$NAD^+/NADH$ (total)	6.68	3.60
$NADP^+$ (total)	0.069 ± 0.008 ($n=16$)	0.064 ± 0.008 ($n=14$)
NADPH (total)	0.219 ± 0.012 ($n=20$)	0.230 ± 0.012 ($n=12$)
$NADPH/NADP^+$ (total)	3.17	3.59

The values are means ± SEM of four measurements in n dogs. The cytoplasmic $NAD^+/NADH$ ratio was calculated using 1.11×10^{-4} for the LDH equilibrium constant and mitochondrial $NAD^+/NADH$ using 3.87×10^{-6} M for the GDH equilibrium constant. This corresponds to the 'free' nucleotide ratio g since there is significant binding of the reduced form.

[a] $= p < 0.05$.

[b] $= p < 0.01$.

ratio in the cytosolic compartment but a 70% increase in the mitochondrial ratio. From this, the reductive change in the total nucleotide is due entirely to a cytoplasmic change. The mitochondrial change in fact is towards oxidation.

From the spectroscopic calculations 85% of the pyridine nucleotide is extramitochondrial. The reciprocal change in the two compartments is consistent with a net change of 0.30 mmoles/kg dry weight in NADH, close to that actually found.

The total level of 2.3 mmoles/kg dry weight found in the dog can also be compared with the value calculated from the absorption data of 0.141 mmoles/kg wet weight. Since 20% of tissue is dry weight this corresponds to a value of 0.7 mmoles/kg dry weight. The epithelial cell layer comprises only 50% of the mass of the stripped frog mucosa and mucus cells contain only about 2/3 of the tubular (oxyntic) cell content which allows an estimated value of close to 2 mmoles nucleotide per kg dry weight oxyntic cell mass in the frog which is quite comparable to the value found for dog parietal cell regions.

Discussion

From the data presented in the frog and the dog, a consistent picture emerges in terms of what happens to the total pyridine nucleotide with onset of secretion. Thus a small component becomes reduced ranging from about 4% in the frog to about 10% in the dog, measured by quite different techniques.

Moreover, calculation of the cytosolic and mitochondrial distribution of the change in the NAD^+/NADH ratio shows that the reductive change occurred exclusively in the cytoplasm. On the other hand the mitochondrial compartment became relatively oxidized. The ratios calculated are consistent with those found for other tissues [6].

Unfortunately the flavine nucleotides and the cytochromes are not amenable to estimation by the cycling procedures. However, just as the changes in the dog tissue for pyridine nucleotides appear to match these measured spectroscopically in the frog, it seems reasonable to assume that reductive changes occur in FAD and the cytochromes in dog gastric mitochondria with onset of acid secretion.

Since, therefore, the intramitochondrial NAD^+/NADH increases and the FAD/$FADH_2$ decreases, a cross-over occurs between these components of the redox chain.

The oxidation of mitochondrial NADH would be consistent with the fall in the phosphorylation potential, ATP/ADP·Pi as found in both frog and dog [3, 18]. The problem is that the other components of the chain become reduced.

A reduction of NAD and cytochrome *c* in the mitochondrial respiratory chain with work load (muscle contraction) utilizing ATP has been described in insect muscle mitochondria [7]. Here the effect has been explained by a low mitochondrial permeability to metabolic anions and

activation of NADH-dependent isocitrate dehydrogenase. This action, activation of substrate oxidation, supersedes the oxidation of the respiratory chain expected from the decline in phosphorylation potential. It is clear from these data that an anomalous reduction of the respiratory chain does not disprove ATP utilization for work done by a tissue.

A similar situation may exist in part in the stomach. Thus, the largest changes found in metabolite levels in the dog are found in the Krebs cycle intermediates [16]. The effect of burimamide and histamine are also best explained metabolically as being due to substrate mobilization (whether glucose in the dog or additionally fatty acids in the frog) since no electron flow is demonstrable by amytal at FAD and below in the presence of burimamide. This interpretation has been suggested previously [8] and the data are quite similar to those found with theophylline addition to frog mucosa made resting by extensive washing [10].

The involvement of mitochondrial reactions is essential in any mechanism of H^+ secretion. For example, using substrate as a direct H^+ donor we may have

$$\text{lactate} \rightarrow \text{pyruvate} + 2H^+ + 2e.$$

If the protons are secreted and the electrons find their way to O_2, the H^+/O ratio of 2 is obtained provided the pyruvate is excreted and not metabolized. Any direct substrate reaction is subject to the same limitation. If the product of this type of reaction is not excreted but metabolized, the maximal H^+/O ratio is much less than 1, contrary to observation.

Apart from the generation of ATP, mitochondrial metabolism may be directly involved in the reduction of a cytoplasmic H^+ donor and in the reoxidation of a cytoplasmic electron acceptor. The presence of a cross-over between NAD^+ and FAD is not compatible with the utilization of only ATP as a product of mitochondrial metabolism. However, such a finding as well as the reduction of the subsequent redox chain does not exclude ATP utilization.

From the cross-over data it is possible to formulate a specific hypothesis for movement of reducing equivalents into and out of gastric mitochondria. Movement of reducing equivalents out of the mitochondria presents no energetic problem since there is a higher oxidation state of cytoplasmic as opposed to mitochondrial diphosphopyridine nucleotide. Malate would be an appropriate H^+ shuttle, oxidation of malate in the cytoplasm producing cytoplasmic NADH.

This NADH could react with a plasma membrane H^+ transport

system donating H^+ to the lumen, or to a second pump such as the $(H^+ + K^+)$-ATPase [5, 15]. The electrons pass to an acceptor such as dihydroxyacetone phosphate and with $2H^+$ from the cytoplasm, α-glycerol phosphate is formed. This in turn reacts with the flavin linked α-glycerophosphate dehydrogenase as in the α-glycerophosphate cycle [19]. In accord with this possibility there is a rise in malate and α-glycerophosphate with onset of secretion in dog mucosa [16].

This type of reaction sequence may account for a decrease in the cytoplasmic NAD^+/NADH ratio coupled to an increase in mitochondrial NAD^+/NADH ratio associated with a fall in the $FAD^+/FADH_2$ ratio.

In general, therefore, these data suggest the possibility that a reaction may be involved in gastric acid secretion other than, or in addition to, utilization of ATP. Data with isolated vesicles [15] as well as the fall in phosphorylation potential [3, 18] implicate ATP in the process. Thus, the final answer to the problem of H^+ secretion in the stomach may eventually be found in a combined redox and ATP-driven mechanism.

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