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Effects of azide on gastric mucose

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Sodium azide, a classical inhibitor of cytochrome oxidase, is an effective inhibitor of gastric acid secretion in bullfrog and skate gastric mucosae at low concentrations. While a portion of the oxygen uptake in these tissues is sensitive to azide ($K_I < 2$ mM), there remains a large fraction (25–60%) with a K_I more than 10 times this value, suggesting the presence of a second oxidase. The spectra of cytochromes *c* and *b* change with oxygen-nitrogen alternation in the presence of high azide concentrations which essentially eliminate the reactivity of cytochrome oxidase. In both species two additional components are observed in the spectra. The first has a peak at 590 nm, is not the cytochrome oxidase-CO complex, is fully reactive in the presence of azide and accounts for the asymmetry of the oxidase peak. The second is a component at 557 nm which can only be separated from cytochromes *c* and *b* by spectral deconvolution, and seems to react in a manner similar to cytochrome *c*. It is suggested that the 590 compound may be the alternate cytochrome oxidase.

Introduction

The secretion of acid by the gastric mucosa in many species is known to be an aerobic process, with secretion decreasing to zero under anaerobic conditions [1]. The gastric mucosa is known to contain a cytochrome system [2,3] which is presumably responsible for generation of the large amount of energy necessary to drive acid secretion [4]. It was therefore not surprising that cyanide (CN^-) and azide (N_3^-) were found to be potent inhibitors of gastric acid secretion, since these compounds are classical inhibitors [5] of the terminal oxidase. Inhibition by these agents was therefore interpreted as inhibition of cytochrome oxidase, blocking respiration and producing a state not unlike anoxia, in which acid secretion is inhibited by lack of respiratory energy.

Recent experiments have cast doubt on this interpretation. We [6] found that acid secretion in this tissue is only partially inhibited by high concentrations of carbon monoxide (CO), which is a competitive inhibitor of cytochrome oxidase in most systems. Moreover, there were indications from observations of the electric potential difference across the tissue [7] that the azide-inhibited state is not identical to the anoxic state, although both show no acid secretion. These observations lead to the hypothesis that the inhibition of acid secretion by CN^- and N_3^- might not be due to their effects as inhibitors of cytochrome oxidase, but rather to other activities of these compounds, such as the uncoupling which can be demonstrated in inhibitor-insensitive systems [8,9]. The present experiments were designed to investigate the inhibitor resistance of the gastric mucosa of two species.

Some of these data have been presented in preliminary form [10–12].

Materials and Methods

Tissue preparation and solutions. Bullfrogs (*Rana catesbiana*) were purchased from suppliers and stored at room temperature in running deionized water until used (usually less than 7 days). They were killed by decapitation and pithing, the stomach removed, and cut open along its lesser curvature. The heavy muscle layer was removed by blunt dissection under cold, oxygenated Ringer's solution, and the resulting mucosa mounted as a flat sheet across the opening of an Ussing-type chamber. The tissue was bathed with the solutions shown in Table I. The serosal solution was augmented with glucose (10 mM) and β-hydroxybutyrate (10 mM) as metabolic substrates [13], and histamine (0.1 mM) as secretagogue. Skates (*Raja erinacea*) were collected by ground trolling in the Gulf of Maine, transported to the

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TABLE I

Solutions employed

Dissolved in deionized water. Serosal solution is an approximation to the plasma of the organism; mucosal solution similar but unbuffered, with NaCl added to maintain Na^+ concentration. All values are in mEq/l.

Component	Frog gastric mucosa		Skate gastric mucosa	
	mucosal	serosal	mucosal	serosal
Na^+	104	104	251	251
K^+	4	4	10	10
Ca^{2+}	1	1	5	5
Mg^{2+}	0.8	0.8	2	2
Cl^-	98.5	86	275	244
HCO_3^-	–	12.5	–	30
HPO_4^-	–	–	–	1
Urea	–	–	350	350

laboratory and kept in 'live cars' attached to the dock until used. They were killed by cervical transsection, the stomach removed, dissected and mounted as above. All solutions were gassed and circulated with 90% O_2/10% CO_2 to achieve maximal secretory rate [14,15]. Under these conditions, acid secretion, transepithelial potential difference or short circuit current, and transepithelial resistance are stable in both species for many hours at room temperature in the absence of intervention.

Spectrophotometric experiments. For spectrophotometric experiments, the tissue was mounted in a plexiglas chamber exposing 4.91 cm^2 of tissue to each solution and arranged so that light could be passed through the tissue for optical observations, using the multiple reflectance system [16] to increase optical sensitivity. Unbuffered mucosal solutions were employed to allow measurment of acid secretory rate by the pH-stat method [17], and 10% CO was added to the gasses to inhibit the reactivity of residual hemoglobin [2]. The spectrophotometer [18] can be operated in either the dual-wavelength [19] mode, in which the kinetics of up to four cytochromes can be followed as a function of time, or in the dual-differential scanning mode [18], which produces a difference spectrum. In each experiment, the dual-wavelength mode was used to monitor the tissue response, while spectra were obtained at selected points.

Since the spectra are recorded digitally (every 0.25 nm) various manipulations are possible, such as deconvolution of the components of a composite asymmetrical peak. In this technique, the assumption is made that the composite peak is the result of two components with symmetrical absorption bands which differ in absorbance maximum wavelength and may differ in spectral intensity and/or bandwidth. One can reconstruct the spectrum of the major component by assuming that the side of the composite peak away from the minor peak is an accurate measure of the major peak alone, reflecting that side of the peak to the other side to generate a spectrum of this major peak. Subtracting this reconstructed peak from the recorded composite peak produces a spectrum of the minor component. If this minor peak is also asymmetrical, the process can be repeated.

Oxygen consumption experiments. For measurement of oxygen consumption, a different chamber system was used. Because of difficulties with O_2 absorption into various plastics tried, this chamber was constructed of stainless steel, with dimensions chosen to maximize the tissue surface to chamber volume ratio for best sensitivity. The chamber was equipped with fluid reservoirs, gassed and stirred by an air-lift system. The chamber could be isolated from the reservoirs by clamping the hoses with hemostats. When this is done, tissue respiration reduces the O_2 concentration in the chambers, which is recorded with a pair of Instec Mod. 125/05 Clark-type [20] membrane covered oxygen electrodes, which produce a current of about 100 nA in 100% O_2 at 20 °C; the electrodes thus consume negligible O_2 in the course of an experimental run. These small currents were transformed into voltages by an active current to voltage converter, producing a voltage which was recorded against time on a dual channel recorder. To provide stirring when the chambers were isolated, internal plastic-coated stirring magnets were magnetically coupled to a pair of external rotating magnets through the thin stainless steel walls. Water at 20 °C was circulated through an external copper coil in contact with the chamber body for temperature control. Since acid secretion was not to be measured, buffered (serosal) solution was used on both surfaces. With the stainless steel chambers, electrical measurements were not attempted.

For each tissue, an initial respiratory rate determination was made in the absence of azide, and at least 20 min after mounting and stimulating the tissue with histamine (0.1 mM) and β-hydroxybutyrate (10 mM). Making this measurement involves clamping the connections to the reservoirs and thus isolating the chambers, allowing the oxygen electrodes to indicate the decrease in O_2 concentration due to tissue respiration. This can be converted to the respiratory rate with a knowledge of the volume of the fluid in each chamber half (6.25 ml), the solubility of O_2 in Ringer's at 20 ° (31.0 μl/ml fluid) [21], the area of the tissue exposed to each solution (7.07 cm^2) and the slope of the recorded line. The O_2 concentration was never allowed to drop below 0.7 atm, since acid secretory rate has been shown to be independent of O_2 concentration only above this level [22]. The constency of respiration over this range implies that this level of O_2 is also sufficient for maximum respiration. Following the control measurement the chamber was unclamped, allowing the O_2 concentration to re-equilibrate with the gas mixture. N_3^- was added to both sides of the tissue in graded steps to a

maximum of 25 mM (frog) or 100 mM (skate), allowing at least 20 minutes of equilibration (hoses unclamped) after each addition before the subsequent measurement. Not all concentrations were run on the same tissue. Before and after the experiment, the electrode zero was determined by gassing with 90% N_2/10% CO_2, which usually resulted in negligible oxygen electrode output.

Results

Azide inhibition of acid secretion

The tissues mounted for spectrophotometry were monitored for acid secretory rate in control and in azide-inhibited conditions. As shown in Table II, low concentrations of N_3^- in the serosal bathing solution markedly inhibit gastric acid secretion, the inhibition at a given concentration is higher in frog than in skate, and acid secretion can be completely abolished by sufficient N_3^- (5 mM in frog, 25 mM in skate).

Oxygen electrode experiments

A series of oxygen electrode traces were obtained on 10 frog gastric mucosae, using azide concentrations between 0 and 25 mM. The mean control respiratory rate for these tissues was 2.25 ± 0.40 μmol/cm² per h from the mucosal and serosal solutions combined. To remove variability between tissues, the rates obtained in N_3^- were normalized to the control measurement for

TABLE II

Effect of azide on acid secretory rate of frog and skate gastric mucosa

Data are mean ± S.E., for (N) tissues, and represent terminal 15 min of a 1 h period in the indicated condition. Probabilities by t-test: ns, $P > 0.05$; *, $0.05 > P > 0.01$; **, $P < 0.01$. Note different azide concentration range for the two species.

Azide concn.	None	0.5	1.0	5.0
Frog:				
Rate (μEq·cm^{-2}·h^{-1})				
Mean ± S.E.	2.98 ± 0.23	0.91 ± 0.21	0.05 ± 0.05	0.00 ± 0.00
(N, P [a], P [b])	(13, –, **)	(10, **, **)	(8, **, ns)	(7, **, ns)
% of Control				
Mean ± S.E.		30.7 ± 7.33	1.72 ± 1.84	0.00 ± 0.00
(N, P [a], P [b])		(10, **, **)	(8, **, ns)	(7, **, ns)
Azide concn.	**None**	**1.0**	**5.0**	**25.0**
Skate:				
Rate (μEq·cm^{-2}·h^{-1})				
Mean ± S.E.	2.25 ± 0.31	0.26 ± 0.11	0.15 ± 0.08	−0.01 ± 0.00
(N, P [a], P [b])	(15, –, **)	(7, **, ns)	(9, **, ns)	(5, **, ns)
% of Control				
Mean ± S.E.		11.4 ± 4.6	14.6 ± 10.3	−0.8 ± 0.3
(N, P [a], P [b])		(7, **, *)	(9, **, ns)	(5, **, ns)

[a] Comparison with control; if different, inhibition has occurred.
[b] Comparison with zero; if different, inhibition is incomplete.

TABLE III

Effect of azide on oxygen uptake of frog gastric mucosa

Calculated from the slope of recording of oxygen electrode output vs. time. Electrodes calibrated at 90 and 0% O_2 by equilibration with known gas mixtures; slopes determined above 70% saturation. Data are mean ± S.E. for N experiments, with significance of difference from control (100%) indicated as in Table II; all are different from zero ($P < 0.01$) except the 25 mM points where $0.01 < P < 0.05$.

Azide (mM)	O_2 consumption relative to control			Ratio (muc/ser)	N
	mucosal	serosal	total		
0.0	1.000	1.000	1.000	0.815 ± 0.079	10
0.1	0.906 ± 0.062 ns	0.867 ± 0.018 ns	0.888 ± 0.019 ns	0.829 ± 0.242 ns	2
0.2	0.715 ± 0.028 ns	0.640 ± 0.034 ns	0.675 ± 0.003 **	0.889 ± 0.261 ns	2
0.5	0.637 ± 0.057 **	0.576 ± 0.055 **	0.597 ± 0.027 **	0.833 ± 0.110 ns	5
1.0	0.576 ± 0.032 **	0.430 ± 0.074 **	0.493 ± 0.045 **	1.150 ± 0.245 ns	6
2.0	0.436 ± 0.036 **	0.408 ± 0.049 **	0.434 ± 0.039 **	0.969 ± 0.164 ns	5
5.0	0.362 ± 0.112 *	0.387 ± 0.123 *	0.348 ± 0.030 **	0.873 ± 0.340 ns	3
10.0	0.429 ± 0.049 **	0.318 ± 0.052 **	0.369 ± 0.041 **	1.286 ± 0.219 ns	5
15.0	0.281	0.149	0.210	1.636	1
25.0	0.342 ± 0.060 **	0.224 ± 0.046 **	0.282 ± 0.073 **	1.604 ± 0.438 ns	5

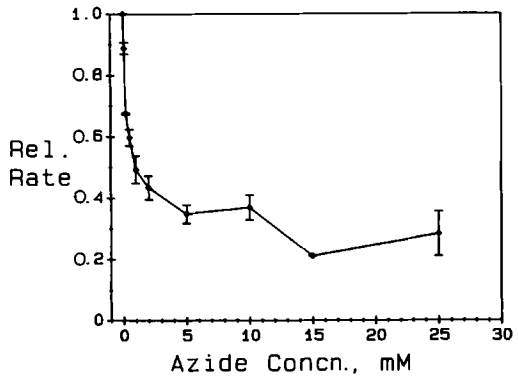

Fig. 1. Effect of azide on respiration (sum of both surfaces, mean ± S.E.) of frog gastric mucosa, relative to the control value for that issue. Note the sensitivity of part of the rspiration and the insensitivity of the remainder.

total O_2 consumption relative to control as a function of N_3^- concentration. It is clear without further analysis that a portion of the tissue respiration is quite sensitive to N_3^-, while there is another portion, amounting to some 30% of the total, which is very insensitive to N_3^-. At low azide concentrations, the respiration from the serosal surface is more inhibited than that from the mucosal surface.

Similar results are obtained in skate. In 72 measurements on 11 tissues, N_3^- concentrations from 0.1 to 100 mM were tested. Low concentrations (to 1 mM) inhibit O_2 consumption by 25%, while in higher concentrations (to 100 mM) the respiratory rate remains above 40% of control. As in the frog, there is no evidence of respiratory stimulation; in 0.1 mM N_3^-, the lowest concentration employed, the respiratory rate was 80.4 ± 6.9 for the serosal surface and 98.3 ± 7.2 for the mucosal surface ($N = 5$). There is more variability in the skate data than in the frog, but the faction of respiration remaining in the presence of N_3^- is greater in skate, and more inhibition is seen of the O_2 uptake from the serosal surface, as in frog.

Spectrophotometric observations

Fig. 2 shows a series of spectra taken from the same frog gastric mucosa with increasing azide concentrations. These spectra show those cytochrome components free to react with O_2 at that N_3^- concentration. At all N_3^- concentrations, there are cytochromes which respond to the removal of O_2. With increasing N_3^- concentration, the peak due to cytochrome oxidase (602.5 nm) is reduced to a very small magnitude, showing nearly total inhibition of this cytochrome. Cytochrome *c* is less changed by azide, with about 50% of its activity remaining in 1 mM N_3^-, a concentration at which acid secretion is eliminated. At 5 mM N_3^- cytochrome *c* is largely unreactive, but a cytochrome *b* component remains almost completely reactive, which implies that even in these conditions O_2 is capable of oxidizing cytochrome *b* by some route.

Three other features are demonstrated by these spectra. First, spectra numbers 1 and 2 differ only in that no. 1 uses the oxidized 'overshoot' state for its reference while no. 2 uses the secretory steady state. The cytochrome *c* peak is of lower height in no. 2 than in no. 1, while the other standard components have not appreciably changed. This implies that cytochrome *c* is partially reduced in the secreting steady state, as previously reported [3,23]. Second, the asymmetry of the cytochrome oxidase peak in spectrum no. 1 would seem to be due to a shoulder on this peak at about 590 nm, and this shoulder is reduced in intensity when the reference is the steady-state condition, showing that this component is considerably reduced in the secreting steady state. Finally, the cytochrome *c* peak does not lie at 550 nm, but is displaced toward longer wavelengths. This is a consistent observation.

Fig. 3 shows a similar example from the skate gastric mucosa. Compared to frog, the control spectrum (no. 1) in this tissue has less cytochrome *c* relative to *b* and the oxidase, and larger asymmetries in the observed peaks. The (N_3^- + O_2) minus O_2 spectrum (no. 2) shows reduction of some of the cytochrome *c* (550) and most of the cytochrome oxidase (602.5), but only a minor amount of cytochrome *b* (664). The resulting cytochrome oxidase peak is quite symmetrical. When the N_2–O_2 spectrum is obtained in the presence of N_3^- (no. 3), some of the cytochrome *c*, most of the cytochrome *b* and a small amount of cytochrome oxidase are free to react. Most interesting is the appearance of a peak at 590 nm which can be oxidized in the presence of N_3^-, and is respons-

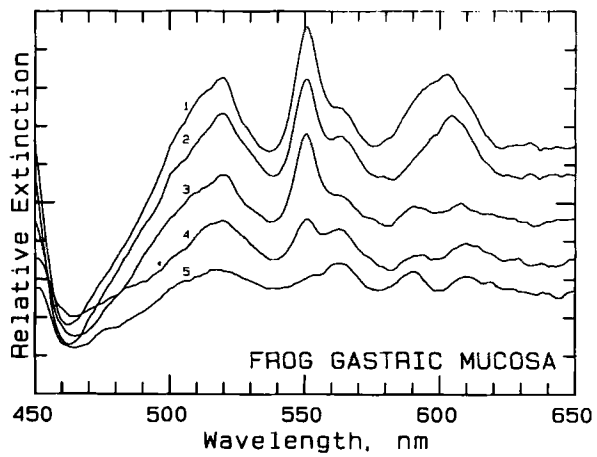

Fig. 2. Difference spectra of the chambered frog gastric mucosa. All are N_2–O_2 difference spectra. Spectra numbers 1 and 2 are in the absence of azide; for no. 1 the oxidized reference spectrum was at the peak of the overshoot, while for no. 2 the oxidized reference was the secreting steady state. Spectra no. 3, no. 4 and no. 5 are in the presence of 0.5, 1 and 5 mM azide, respectively. Peaks are identiifed as follows: 465 nm (trough), flavoprotein; approx. 520 nm, fused β-bands of all cytochromes; 550 nm, cytochrome *c*; 564 nm, cytochrome(s) *b*; 602.5, cytochrome oxidase (cytochrome $a + a_3$).

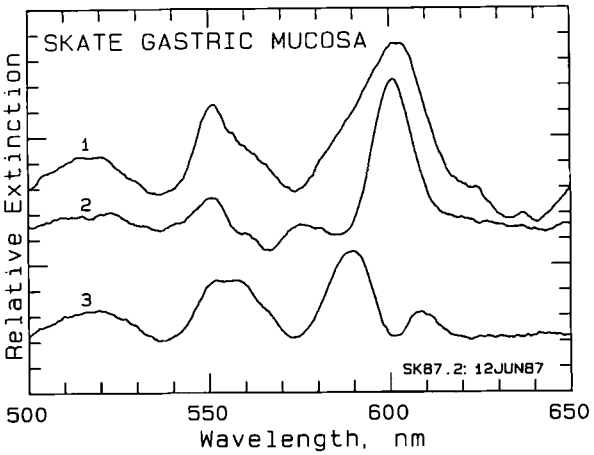

Fig. 3. Difference spectra of the chambered skate gastric mucosa. Spectrum no. 1 is the N_2-O_2 difference spectrum under control conditions. Spectrum no. 2 is the same tissue in 5 mM NaN_3 and oxygen, minus the oxygenated control, and therefore shows only those components which are reduced in the presence of azide. Spectrum no. 3 is the N_2-O_2 difference spectrum in the presence of 5 mM NaN_3, and shows those components which are responding to O_2 in the presence of azide. Peak identifications as in Fig. 2.

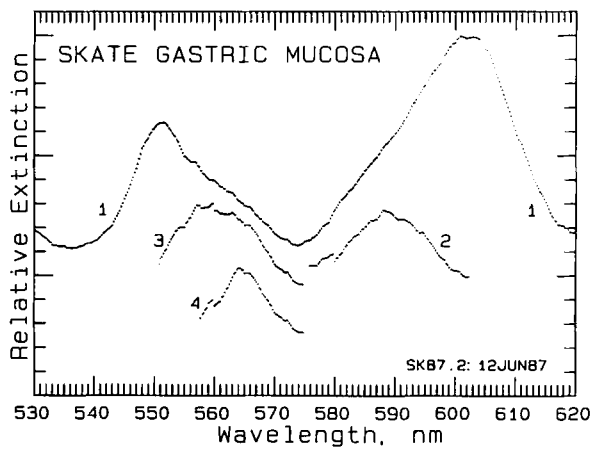

Fig. 5. A difference spectrum of skate gastric mucosa and some deconvolutions. Spectrum no. 1 is the N_2-O_2 difference spectrum (Spectrum no. 1 of Fig. 4) plotted on an expanded scale, with each dot representing one digital reading (0.25 nm). Spectrum no. 2 is the result of subtracting the 602.5 peak reconstructed from the long-wavelength side. Spectrum no. 3 is the result of subtracting the 550 peak reconstructed from the short-wavelength side, while spectrum no. 4 is a double deconvolution, generated by subtracting the peak at 557 nm reconstructed from the long wavelength side from spectrum no. 3.

ible for the asymmetry of the cytochrome oxidase peak in the control spectrum.

The cytochrome a_3-CO complex has an absorption maximum at 590 nm, which could be the source of this component, since the gas mixtures contain 10% CO. Therefore, some experiments were done in the absence of CO. An example is shown as Fig. 4. The control spectrum still shows the marked asymmetry of the cytochrome oxidase peak. When this peak is deconvoluted from the long-wavelength side, the reason for the asymmetry is seen to be a peak at 590 nm. Since this tissue was never exposed to CO, the resulting 590 peak must be characteristic of the reduced-minus-oxidized spectrum of the tissue itself.

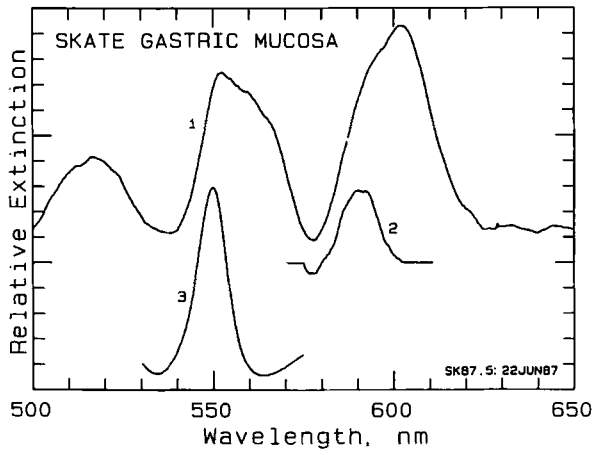

Fig. 4. Difference spectra of skate gastric mucosa in the complete absence of CO. Spectrum no. 1 is the N_2-O_2 control spectrum; the cytochrome oxidase peak is still asymmetrical, which could not be due to the cytochrome a_3-CO complex under these conditions. Spectrum no. 2 is the result of deconvoluting this peak by removal of the 602.5 nm component reconstructed from the long-wavelength side. Spectrum no. 3 is the α-peak of authentic cytochrome *c*, which falls exactly on 550.0 nm, as it should.

Both frog and skate spectra show a displacement of the cytochrome *c* α-peak, normally at 550.0, to longer wavelengths and an asymmetry of this peak apparently due to other components on the long-wavelength side; the asymmetry is particularly marked in skate. We expect cytochrome *b* (α-peak 564) to appear as a shoulder in the cytochrome *c* peak, but the degree of asymmetry and the shift of peak location are too large to admit this as the sole interpretation. Within the assumptions underlying the deconvolution technique, these peaks can be resolved into their components. Fig. 5 shows a control spectrum (no. 1) of skate gastric mucosa and some deconvolutions of that spectrum. A first deconvolution (no. 2) by subtracting the 602.5 cytochrome oxidase peak produces the peak at 590 previously shown, while subtraction of the 550 nm cytochrome *c* peak gives a residual peak (no. 3) with a maximum at 557, which is itself asymmetrical. Further deconvolution of this peak by subtraction of the reconstructed 557 nm peak gives a smaller peak (no. 4) at 564, appropriate to cytochrome *b*. It would thus appear that the spectrum observed under control conditions contains cytochromes with maxima at 550 (cytochrome *c*), 557 (?), 564 (cytochrome *b*), 590 (?) and 602.5 (cytochrome oxidase). By analogy to their nearest neighbors, we will call the unidentified components *c*-557 and *a*-590, respectively. Similar manipulations of spectra from frog gastric mucosa produce similar results, but the contribution of *c*-557 and *a*-590 to the overall spectra is not as great in this species.

TABLE IV

Azide effects on N_3–O_2 difference spectra of mucosae from skate and frog

Mean (±S.E. for N tissues) of the peak height in the highest azide concentration tested as a percentage of the height of that peak in the absence of azide. Flavoprotein and cytochromes *c* and *a* measured directly from spectra; *c*-557, *b* and *a*-590 from deconvolutions. Significance of the difference between observed ratio and 0 (complete inhibition) and 100 (no inhibition) is indicated with symbols as in Table II.

Pigment	(nm)	Skate, 25 mM azide ($N = 6$)			Frog, 5 mM azide ($N = 7$)		
		% of control	Sig. vs.		% of control	Sig. vs.	
			0	100		0	100
fp	(465)	39.9±18.1	ns	*	35.9± 0.9	**	**
c	(550)	37.3± 5.9	**	**	14.3± 5.8	*	**
c-557	(557)	80.6± 9.8	**	ns	34.7± 6.8	**	**
b	(564)	81.7±16.6	*	ns	38.6± 7.9	**	**
a-590	(590)	137.2±31.5	**	ns	39.5±29.6	ns	ns
a	(602.5)	11.0± 1.4	**	**	−7.6± 7.4	ns	**

Table IV presents a summary of the spectrophotometric data for the highest azide concentration used, expressed as percent of control response. At the high N_3^- concentration shown, only 11% of the skate cytochrome oxidase responds to O_2, but cytochromes *b*, *c*-557 and *a*-590 are more than 50% oxidized by O_2. The frog gastric mucosa shows many of these same effects, although not so dramatically, and at lower concentrations. At this high concentration cytochrome oxidase is completely inhibited while a significant portion of the other components show redox changes. The 590 pigment is variable; while it appears strongly in individual spectra, the variability produces a lack of significance in these data.

Discussion

If O_2 is required for gastric acid secretion [1], the elimination of oxygen consumption should inhibit secretion. Sodium azide has long been known as an inhibitor of cytochrome oxidase [5], and is an effective inhibitor of acid secretion in the frog gastric mucosa, which is consistent with this view. However, the lack of inhibition of acid secretion by carbon monoxide, another cytochrome oxidase inhibitor [24], suggests that the inhibition by N_3^- might not be through elimination of tissue respiration, a view which is strengthened by the observation that the cytochrome system in both species still interacts with O_2 in the presence of high CO/O_2 ratios (Ref. 24; see also unpublished observations).

The oxygen-uptake experiments confirm this suspicion. Only 25% of the respiration of frog gastric mucosa is inhibited by 1 mM N_3^-, while acid secretion is abolished. Higher N_3^- concentrations produce more respiratory inhibition, but even at the highest concentration tested some 30% of respiration remains. Similar results obtain in the skate gastric mucosa. This tissue has an acid secretory rate less sensitive to N_3^-, and a respiratory rate less inhibited by N_3^-, while likewise showing lack of inhibition by CO [24]. Thus in two species, N_3^- is shown to be an incomplete inhibitor of O_2 uptake, while acid secretion is resistant to CO.

This suggests that there may be two oxidases present. We expect an azide-sensitive cytochrome oxidase, and have presented spectral evidence for the existence of the usual cytochrome oxidase peak in both frog [3] and skate [10]. The present data suggest that another azide-insensitive oxidase may be present; this oxidase would seem to have a resistance to N_3^- which is significantly higher than cytochrome oxidase. Dixon [25] plots of the data from the frog gastric mucosa allow estimation of the K_I of the oxidase for N_3^-. Such a plot is shown as Fig. 6 and shows two lines of apparent inhibitor affinity, corresponding to a low and a high K_I. In intact tissue, one must take the K_I's as maximum approximations, but the presence of two enzymes seems clear. The

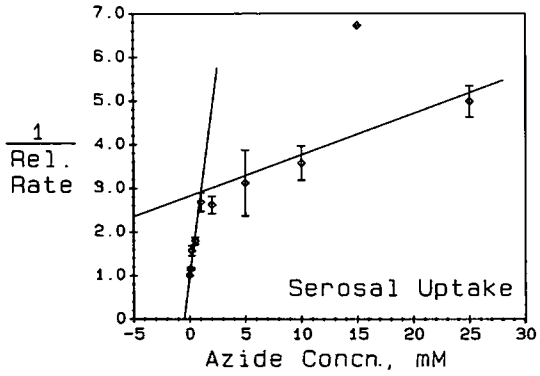

Fig. 6. Dixon plot of the effect of azide on the respiration from the serosal surface of the frog gastric mucosa. Points are the means at that concentration; error bars are ±1 S.E. The two lines are fit by least squares to points from 0.1 to 1 mM, and from 5 to 25 mM, with the point at 2 mM considered transitional and not used to fit either line. From the intercepts of these lines, the K_I for azide is calculated as 0.55 and 29.8 mM in these two ranges. A similar plot for the mucosal surface yields a 'sensitive' K_I of 2.18 mM and an 'insensitive' K_I of 132.7 mM.

low-K_I component is probably cytochrome oxidase, while the high-K_I component remains to be identified.

A similar analysis for the skate gastric mucosa produced unsatisfactory plots, partially due to the smaller fraction of respiration which is very sensitive to N_3^-, and partially due to greater variability in the data. Dixon plots for N_3^- concentrations of 5 mM and above (the high-K_I region) give reasonable lines ($r = 0.60$ for the serosal surface and $r = 0.46$ for the mucosal surface, $N = 44$ for each), and K_I's of 60.3 and 96.8 mM for the serosal and mucosal surfaces, respectively, which is in reasonable agreement with the data from the frog, given the possible errors in both data sets, and certainly very much higher than the expectation for cytochrome oxidase.

Another evidence for the existence of an alternate cytochrome oxidase comes from the spectral data. If cytochrome oxidase were the only route of interaction of the cytochrome system with O_2, and if cytochrome oxidase were completely inhibited by azide, then in its presence the cytochromes should be completely reduced and show no changes when O_2 is removed or replaced. Under these conditions, the N_2–O_2 difference spectrum should show no cytochrome components. The spectra presented show that while the cytochrome oxidase changes are essentially abolished by 1 mM (frog) or 5 mM (skate) N_3^-, cytochrome *c* and especially a cytochrome *b* component are still showing redox changes with changes in O_2. We thus infer that some other component than the 602.5 nm cytochrome oxidase pigment must be responsible for the interaction with O_2 that allows for the persistance of cytochrome redox changes when the usual cytochrome oxidase is inhibited.

This alternate oxidase might be any component of the cytochrome system which shows redox reactions in the presence of inhibitor, or might not be spectroscopically observable. It seems unlikely that it is one of the other members of the normal cytochrome chain, since these do not react with O_2. Our attention is thus focused on the two novel components present in the gastric mucosa. The component responsible for the peak at 590 nm would seem a likely candidate for the alternate oxidase; it can be demonstrated to undergo redox changes with changes in O_2, it is not inhibited by N_3^-, and its spectral peak wavelength suggests a modified cytochrome oxidase. This component is present in a higher ratio to the other cytochromes in skate than in frog, consistent with the greater resistance of skate respiration and spectral changes to azide inhibition. Thus while not proven to be an alternate oxidase, the properties of '*a*-590' are consistent with that interpretation.

The component called *c*-557 nm is not easy to identify. As with *a*-590, it is present in both species but relatively more abundant in skate, causing the greater asymmetry of the peak in skate than in frog. It would not seem to be cytochrome c_1, since spectra in frog at $-190\,^\circ C$ show that, if anything, the amount of c_1 in this tissue is less than usual, relative to cytochrome *c* [23]. It is possible that it is a second peak of *a*-590, although in this case a greater correlation between the responses of these two peaks would be expected in data such as that in Table IV. The role of this component in electron transport and gastric acid secretion remains to be elucidated.

One difference between these experiments and those with carbon monoxide is that acid secretion is abolished by N_3^- but not by CO. Since N_3^-, but not CO, has been found to be an uncoupling agent in inhibitor-insensitive respiratory systems [8], and since uncoupling is frequently due to the formation of proton-conductive shunts in a membrane, we suggest that the inhibition of acid secretion by N_3^- might be a consequence of this uncoupling activity, and not due to inhibition of energy production per se. We have not been able to demonstrate accelerated oxygen consumption in this tissue with low doses of N_3^-, but neither can we find accelerated oxygen consumption with 2,4-dinitrophenol, a classical uncoupling agent which is known to inhibit gastric acid secretion [26]. There may be many intracellular sites of azide interaction. What is clear is that if azide inhibition of acid secretion were due to inhibition of cytochrome oxidase and consequent abolition of oxidative phosphorylation, we would expect CO to abolish secretion and azide to abolish respiration.

These observations are consistent with a hypothesis previously presented [2,4] for the energetics of gastric acid secretion. In this hypothesis, a standard cytochrome chain exists in the oxyntic cell mitochondria, and is responsible for production of ATP for many metabolic uses. Gastric acid secretion, however, is not driven by this ATP, but by the activities of a second cytochrome chain located in the mucosal facing plasma membrane of these cells, and producing a proton gradient across this membrane in the manner suggested by Mitchell [27]. N_3^- and CN^- might inhibit acid secretion by virtue of their uncoupling activity, or by some other mechanism, but not by inhibition of the terminal oxidase. We suggest that the oxidase for this secretory chain may be *a*-590, and that *c*-557 might be the *c*-type cytochrome in this chain. Further experiments will be necessary to isolate these components and determine their properties.

One would like to extend the oxygen-consumption measurements to include the other classical cytochrome oxidase inhibitors cyanide and CO. This is not practical with intact tissue, since HCN is volatile and its concentration cannot be maintained during gassing, while CO requires hyperbaric conditions to achieve a high CO/O_2 with the high O_2 required [22]. Therefore, such experiments will require the use of isolated cell preparations.

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