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CYTOCHROMES AND GASTRIC ACID SECRETION

A REEVALUATION OF MUCOSAL ACIDIFICATION EXPERIMENTS

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Summary

Using an improved spectrophotometer, we have reinvestigated the report (Hersey, S.J. (1974) Biochim. Biophys. Acta 344, 157—203) that acidification of the mucosal surface of frog gastric mucosa produces a crossover point between flavoprotein and cytochrome *b*, thus identifying a site of energy coupling between the cytochrome and H^+ transport systems. While we find spectrophotometric changes upon addition of HCl to the mucosal solution, we find similar changes upon addition of NaCl without pH change, but no changes when the pH is lowered by substitution of H^+ for Na^+ at constant osmolality. We show that osmolality changes, with consequent alteration in tissue light scattering, are responsible for these effects. Further, we can show that the pH changes used do not inhibit acid secretion, and that one cannot do so without osmolality increase. We conclude that the imputed crossover point is not demonstrated, and that models based on its existence must be revised.

Introduction

The secretion of H^+ by the gastric mucosa of the frog and other species is directly controlled by the availability of O_2. In the absence of O_2, secretion is abolished [2], and the addition of ATP in this condition will not restore secretion, although sufficient ATP seems to enter the cells to do so [3—5]. This, with the spectrophotometric evidence which implicates the cytochrome chain as a direct energy source for active H^+ secretion [6], has led to a search for the point or points along the chain at which energy is removed. The report of Hersey [1] that decreasing the pH of the mucosal surface from 5.0 to 1.8

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(which was presumed to load the pump and decreases its rate) caused spectrophotometric changes interpreted as identifying a crossover point between flavoprotein and cytochrome *b* was thus of great interest.

Hersey reported that, upon the addition of 1 M HCl to the mucosal bathing solution of a chambered bullfrog gastric mucosa to lower the pH to 1.8, cytochromes $a(+a_3)$, *b* and *c* were oxidized with respect to their previous steady state, while flavoprotein was reduced. Unfortunately, the addition of acid in this quantity increases the osmolality of the mucosal solution by over 50%, which could have effects of its own. Accordingly, we have repeated these experiments to control for the osmolality effects, and find that all of the apparent changes in redox steady state can be produced by an osmolality change alone, apparently by changing the light scattering properties of the tissue. Further, we here present evidence which makes it unlikely that lowering the pH to 1.5 has any significant effect on the acid secretory rate of the tissue.

Methods

Bullfrogs (*Rana catesbiana*) were obtained from Mogul-Ed (Oscosh, Wisc.) or West Jersey Biological Supply Company (Wenonah, N.J.) and were kept in running tap water until used. The frogs were killed by decapitation, and pithed, and perfused by ventricular puncture (after opening the hepatic portal vein) to remove blood from the gastric vasculature. The stomach was removed, opened along its lesser curvature, and the external muscles removed by blunt dissection. The resulting mucosa was mounted as a flat sheet between two fluid-filled chamber halves in the multiple-reflectance chamber [7] for spectrophotometric determinations, and in a standard chamber for secretory studies alone. Both chambers were designed to minimize unstirred layers, and exposed an area of 4.91 cm^2 to each solution.

The serosal bathing solution contains (mequiv./l) Na^+, 104/K^+, 4/Ca^{2+}, 1/Mg^{2+}, 0.8/Cl^-, 82.6/HCO_3^-, 12.5/SO_4^{2-}, 6.25/phosphate, 1. The mucosal solution is identical with the exception of the replacement of sodium phosphate and $NaHCO_3$ by 7.25 mM Na_2SO_4. Both solution are gassed and stirred with 10% CO_2/90% O_2, which provides sufficient supplies of both CO_2 [8,9] and O_2 [10] for maximum secretory rate. For spectrophotometric experiments, 10% CO was added to these mixtures to complex the residual hemoglobin [6], and adenosine (0.5 mM) was added to the serosal solution to inhibit muscular contractions [11]. Anoxia was produced by substituting N_2 for O_2 in these mixtures. Unless otherwise stated, the serosal solution also contains histamine (0.1 mM), with β-hydroxybutyrate (10 mM) as substrate.

The spectrophotometer is a modification of the dual wavelength instrument of Chance [12] which can monitor up to 4 pigments during a single biological change, by rapidly altering its wavelength settings [13]. Improved noise figure and long-term stability compared to previous instruments gives greater confidence in the data. Secretory rate was measured at pH 4.5 by the pH-stat technique [14], transepithelial potential difference (*PD*) by fiber-junction KCl/calomel electrodes inserted into the fluid streams [15], and transepithelial resistance by passing a 1-s 20 μA current via Ag/AgCl electrodes remote from the tissue, recording the voltage deflection produced.

Changes in the pH of the mucosal solution were performed in one of two ways: by the addition of 1 ml of 1 M HCl to the mucosal bathing solution (volume 15 ml), or by substituting for the mucosal solution above a solution containing 75 mM HCl replacing an equivalent amount of NaCl. Other experiments were performed in which 1 ml of 1 M sodium chloride, 1 M choline chloride, or 2 M sucrose was added to produce an osmolality change without pH change.

In investigating the effects of mucosal pH on acid secretory rate, the pH was varied by mixing the isosmotic acid mucosal solution above with the normal mucosal solution, and resetting the pH of the titrator. For these experiments, the titrator was filled with a strong titrant (0.1 N NaOH) to avoid dilution errors [16].

Results

Changing the mucosal solution from pH 4.5 to 1.5 by replacement of Na^+ by H^+ at constant osmolality produces no significant change in the recorded cytochrome signals. A typical experiment is shown in Fig. 1. A small drift in the 564-540 signal is seen to continue through the period of the pH change, and no deflection of any signal is seen correlated with this change. For calibration purposes, a 15 min anaerobic pulse establishes the most reduced state of the pigments, while the peak of the overshoot is taken as the most oxidized state [6]. For purposes of tabulation, the signal at 30 min following the pH change is compared to that immediately prior to the change, and expressed as a percentage of the maximum redox change observed between N_2 and O_2.

By contrast, the addition of HCl, which produces a change in osmolality (212 to 345 mosM) causes changes in the spectrophotometer output. The addition of an equivalent amount of NaCl also causes these changes, as seen in the experiment of Fig. 2. Cytochrome *c* changes in a direction corresponding to an

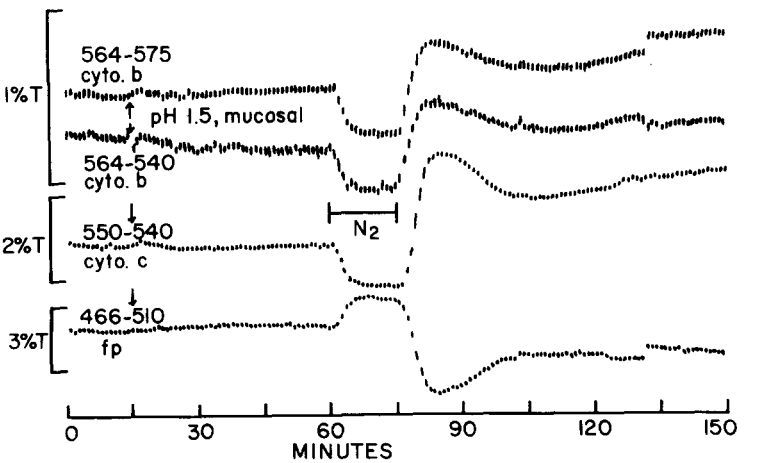

Fig. 1. The effect of decreasing mucosal pH from 4.5 to 1.5 by replacement of Na^+ by H^+. Cytochrome *b* is examined with respect to two different isosbestic points; cytochrome *c* and flavoprotein (fp) at conventional wavelengths. Note no change in cytochrome signals upon mucosal pH change. The subsequent anaerobic period is for calibration. Dwell time 15 s; each pigment examined once per min.

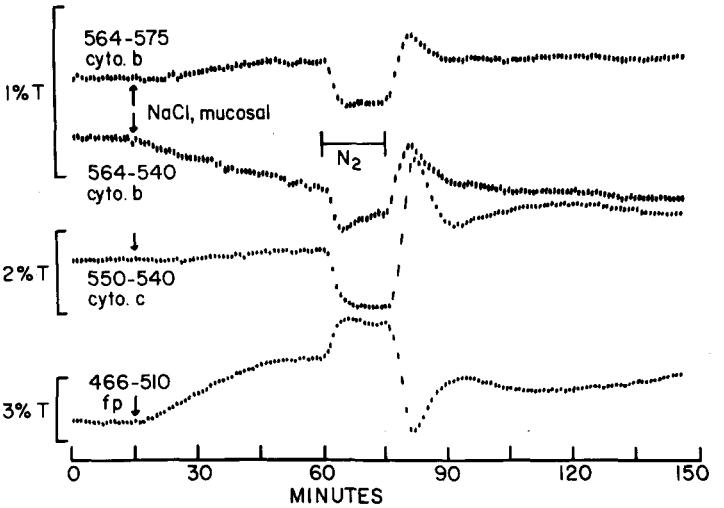

Fig. 2. The effect of increasing the osmolality of the mucosal solution from 212 to 345 mosM by the addition of NaCl. Changes are seen in all components; cytochromes *c* and *b* (564—575) are apparently oxidized, while flavoprotein (fp) is reduced. The apparent reduction in cytochrome *b* as measured by 564—540 makes it clear that these changes are not specific cytochrome changes.

oxidation, while flavoprotein changes in the direction of reduction (oxidized flavoprotein is the species absorbing at 466 nm). Cytochrome *b*, as measured at the conventional wavelengths, (564—575) would be said to be oxidized, but the change with the alternate isosbestic point (564—540) would correspond to a reduction. Clearly, both of these interpretations cannot be correct, and it appears that these changes are due to light scattering changes (see Discussion).

The results of a series of such experiments are presented as Table I. No significant changes were observed when the mucosal pH was changed isosmotically, whereas the addition of HCl or NaCl produces apparent redox changes, in

TABLE I

SUMMARY OF SPECTROPHOTOMETRIC RESULTS

Apparent cytochrome redox changes upon reducing the mucosal solution to pH 1.5 by substitution of H^+ for Na^+, by addition of HCl (66.7 mM) and upon the addition of NaCl (66.7 mM). The numbers are percentages of maximum redox change from anaerobic to aerobic conditions, and are expressed ± standard error of the mean for (*N*) experiments, with + numbers indicating apparent oxidation. The significance of the difference between these numbers and zero is: *, not significant; ** and ***, significant at the 5% and 1% level, respectively. *PD* changes are in mV.

Pigment wavelengths	Cytochrome *a* (+ a_3) (605—625)	Cytochrome *b* (564—575)	Cytochrome *b* (564—540)	Cytochrome *c* (550—540)	Flavo-protein (466—510)	*PD*
To pH 1.5 H^+ for Na^+	+5.2 ± 2.9 (3) *	+5.6 ± 2.5 (4) *	+8.8 ± 10.4 (3) *	+1.0 ± 0.6 (5) *	−0.7 ± 4.0 (5) *	+1.6 ± 1.0 (5) *
To pH 1.5 HCl added	+21.3 ± 7.2 (4) *	±21.1 ± 2.7 (5) ***	−102.8 ± 25.9 (4) **	+3.6 ± 3.7 (7) *	−74.8 ± 26.7 (7) **	+4.4 ± 0.9 (7) ***
No pH change NaCl added	+21.3 ± 11.6 (4) *	+24.7 ± 14.7 (6) *	−80.1 ± 14.1 (3) **	+1.5 ± 4.5 (6) *	−61.8 ± 12.9 (5) ***	−0.3 ± 0.8 (5) *

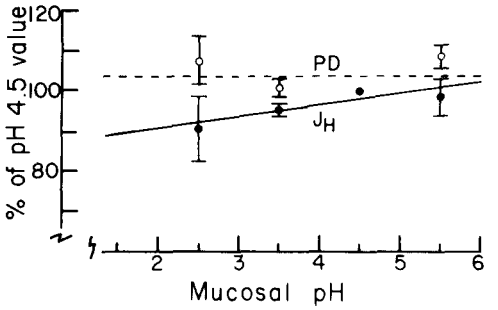

Fig. 3. Acid secretory rate (J_H, solid circles and solid line) and potential difference (*PD*, open circles and dotted line) as a function of mucosal pH. Bars are ± S.E. of the mean; only the points at pH 3.5 (J_H) and 5.5 (*PD*) are significantly different from the value at pH 4.5 (100%). Lines drawn by least squares; neither slope significantly different from zero.

many cases greater than 100% of the maximum redox change. Two additional single experiments, in which 67 mM choline chloride and 133 mM sucrose were added as osmotic agents produce similar results.

In an attempt to answer the question of what the secretory rate might be at pH 1.5 (where it cannot be measured), we have performed a series of experiments in which the mucosal solution was altered from pH 2.5 to 5.5, with the results as presented in Fig. 3. There is no significant trend over this range, and extrapolating these data to pH 1.5 predicts a secretory rate of nearly 90% of that at pH 4.5, a negligible and statistically insignificant decrease. These data thus do not support the contention that the acid secretory mechanism is inhibited at pH 1.5.

In 5 experiments, the pH was returned from 1.5 to 4.5, and titration was resumed. There is considerable adherent acid which confounds the titration for about 5 min, but following this period, the rate is seen to be constant and about the pre-acid level. There was no indication of a rising rate, such as might occur if secretion had been inhibited and was resuming at pH 4.5.

Discussion

In the light of these findings, we need to reevaluate the report of Hersey [1] that changes in mucosal pH can cause changes in cytochrome redox states. We have seen that changes in mucosal pH alone do not alter the spectrophotometric signals, but that changes in mucosal osmolality, whether produced by the addition of HCl, NaCl, or other osmotic agents, produce the changes reported. The explanation that these events are due to an increased load on the H^+ secretory mechanism, and consequently identify a crossover point, must therefore be abandoned.

We can understand the changes observed on the basis of the expected effects of hyperosmotic solutions, which would be expected to produce a decrease in cell water content, and thus in cell size, even in a tissue which is relatively immune to mucosal solution composition. It is not possible to predict accurately the effect of cell size on the light scattering properties of the tissue, since this depends strongly on the geometry. It is to be expected, however, that

one effect of decreased cell size might well be a decrease in the mean optical path length through the tissue, which would consequently decrease the spectrophotometric signal, with the largest decrease at the pigment peaks where the specific absorption was highest and the smallest absolute change at the isosbestic points. Thus, a decrease in mean path length would cause a decrease in the peak heights of the respiratory pigments.

Since the cytochromes show their characteristic absorption maxima in the reduced state, such a decrease in spectral intensity would be interpreted as an oxidation. For flavoprotein, which has a maximum at 466 in the oxidized state, a decrease in intensity would be recorded as a reduction. Thus, a uniform decrease in mean light-path length through the tissue, such as might occur with osmolality changes, will produce a spurious crossover point between flavoprotein and cytochrome *b*, as reported [1]. This analysis is equally valid for experiments performed with dual wavelength instruments and split beam (scanning) instruments. It depends only on the presence in the tissue of a reasonable amount of reduced pigments in the aerobic secreting steady state, which has been well documented [e.g. refs. 1 and 6].

The original experiment was based on the premise that acid secretion is reduced by changing the mucosal bathing solution to low pH. On thermodynamic grounds, there must be a H^+ gradient beyond which the pump cannot operate, and this premise seems a reasonable one until examined further. Our results do not show any direct evidence of a decrease in secretory rate as pH 1.5 is approached, and while it is impossible to measure secretion at pH 1.5, several lines of evidence combined with theoretical considerations make it unlikely that the secretory rate at this pH is decreased compared to higher pH values.

In general, in a wide variety of experimental manoeuvers, any marked decrease in acid secretory rate also produces a change in transepithelial *PD*, a rise in resistance, and a decrease or elimination of the cytochrome overshoot upon reoxygenation. In pH 1.5 mucosal solutions, the *PD* is essentially unaltered from that at pH 4.5 (Table I), there is no rise in resistance, and the overshoot persists (Figs. 1 and 2). Moreover, the addition of the inhibitor SCN^- at low pH produces all of these changes, as it would have upon inhibition of secretion at higher pH. Finally, we have shown that the return from pH 1.5 to 4.5 gives no evidence of the pump slowly resuming activity but rather indicates that its activity is continuing. All of these observations are consistent with the conclusion that gastric acid secretion continues unabated into pH 1.5 mucosal solutions.

This is understandable from theoretical considerations. In frog gastric mucosa, at least 90% of the total active ion transport is H^+ and Cl^-, produced in equal amounts under open circuit conditions [18,19]. As these ions are secreted into the bottom of the crypts, they produce an osmotic force which removes water from the cells to produce HCl which is isosmotic with the cells and, therefore, with the serosal bathing solution. With the solutions used, this primary secretion is therefore 106 mM HCl, which has a pH of 0.97, and this is the solution into which the pump must operate regardless of the higher pH of the bulk solution. The use of a pH 1.5 mucosal solution cannot, therefore, alter this situation. Moreover, any attempt to produce a lower pH than 0.97 in the

bulk solution will necessarily require a hyperosmotic solution (if HCl is used), which would cause its own dilution by water flow from the tissue were it to penetrate to the bottom of the crypts. In addition, it is unlikely that the pH in such a long unstirred layer could be changed much by external addition, especially with the counteracting force of bulk flow of the primary secretion tending to remove ions diffusing inwards.

Thus, while we completely confirm the experimental data of Hersey [1], we can duplicate these apparent cytochrome shifts by osmotic changes alone, and see no cytochrome shifts when pH is lowered without osmotic changes. The original suggestion that the H^+ transport mechanism should be inhibited at a sufficiently low pH is still valid; however, the attainment of pH less than 0.97 requires a hyperosmotic solution, which we have now shown to have effects of its own. Thus, while cytochrome shifts and crossover points might be expected at very low mucosal pH, they cannot be demonstrated by the experiments available, and models which are based on these reported crossover points must be reexamined in this light.

Acknowledgments

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