Ion Transport and the Development of Hydrogen Ion Secretion in the Stomach of the Metamorphosing Bullfrog Tadpole

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ABSTRACT Isolated bullfrog tadpole stomachs secrete H^+ by stage XXIV of metamorphosis, when tail reabsorption is nearly complete. At this stage the PD shows characteristic responses identical to those of the adult. The appearance of HCl secretion correlates well with other studies showing the morphogenesis of oxyntic cells. Prior to the development of H^+ secretion tadpole stomachs maintain a PD similar in polarity and magnitude to that of the adult; i.e., secretory (*S*) side negative with respect to the nutrient (*N*) side. The interdependence with aerobic metabolism appeared to increase progressively through metamorphosis; however, glycolytic inhibitors always abolished the PD. Isotopic flux analysis showed that the transepithelial movement of Na^+ was consistent with passive diffusion, whereas an active transport of Cl^- from *N* to *S* was clearly indicated. Variations in $[Na^+]$, $[K^+]$, and $[Cl^-]$ in the bathing solutions induced changes consistent with the following functional description of the pre-H^+–secreting tadpole stomach. (*a*) The *S* side is relatively permeable to Cl^-, but not to Na^+ or K^+. (*b*) An equilibrium potential for K^+ and Cl^- exists at the *N* interface. (*c*) Ouabain abolishes the selective K^+ permeablity at the *N* interface and reduces the total PD. (*d*) Effects of Na^+ replacement by choline in the *N* solution become manifest only below 10–20 mM. It is concluded that prior to development of H^+ secretion, the tadpole gastric PD is generated by a Cl^- pump from *N* to *S* and a Na^+ pump operating from the cell interior toward the *N* side.

INTRODUCTION

The secretion of HCl by the stomach of several species does not begin until relatively late in embryological development (1–3, see 4). For the fetal rabbit stomach (2, 5) and the chick embryo proventriculus (3) H^+ secretion is first observed at about the same developmental stage at which the oxyntic, or acid-secreting, cell appears. Thus, these studies have provided useful morphological markers associated with the development of H^+ secretion, both

with respect to the general histology and at the fine structural level. However, most previous work in this area has not involved a detailed study of the physiological transport properties of the developing stomach.

Wright showed that, prior to the development of H^+ secretion, the fetal rabbit stomach maintains a transmucosal potential difference, and that a net absorption of NaCl and water occurs, most likely as a result of active Na^+ transport directed from gastric lumen to blood (2). Thus, it appears that cells of the gastric epithelium in this species have certain characteristics similar to absorptive cells further down the gastrointestinal tract; at least this is clear during the early stages of embryological development. There is also evidence to suggest that a similarly oriented Na^+ transport system is present in many adult mammalian stomachs (6–8), but the enormous capacity to secrete HCl in vivo may dwarf the Na^+ absorptive process.

In the present study we have investigated characteristic features of transport processes of the bullfrog tadpole stomach during various metamorphic stages. An active transepithelial transport system for Cl^- was present in all of the developmental stages studied, whereas, H^+ secretion did not appear until very late in metamorphosis. The role of Cl^- transport, the relative interfacial ionic permeabilities, and a Na^+ pump, operating as suggested for mammalian preparations from the mucosal cell to the serosal solution, are discussed in terms of the observed electrical properties of the tadpole stomach.

APPARATUS AND METHODS

Bullfrog tadpoles (*Rana catesbeiana*) obtained from a local supply house (Dahl Co.' Berkeley, Calif.) were used for these studies. The various stages of metamorphosis were taken according to the description of Taylor and Kollros (9). The distinguishing morphological characteristics of the animals as well as the general position and thickness of the stomach for several of the metamorphic stages are shown in Fig. 1. In the early stages of metamorphosis (approximately up to stage XXI) the stomach is a long cylindrical structure about 15–20 mm in length, 3–4 mm in diameter, and is situated at the right side of the body. In later stages, as the tadpole develops, the stomach rotates to the left side of the body and comes to resemble the more distended pouch shape of the adult.

Preparation of the Stomach for the Chambers and Electrical Measurements The tadpole was pithed, the stomach removed, and the lumen washed free of its contents with Ringer solution. The composition of the standard solutions is given in Table I. For open-circuit experiments the tubular stomach was mounted onto a glass chamber as shown in Fig. 2. Usually HCO_3^--buffered Ringer solution was placed on both the nutrient (serosal) and secretory (mucosal) sides of the stomach. However, when H^+ secretion was to be studied, unbuffered saline was used on the secretory side. An air lift mechanism, as shown in Fig. 2, served as a circulating and aerating device with the gas phase being 95% O_2–5% CO_2. The transmucosal potential difference (PD) was measured via porous tip calomel electrodes which were placed into the respective

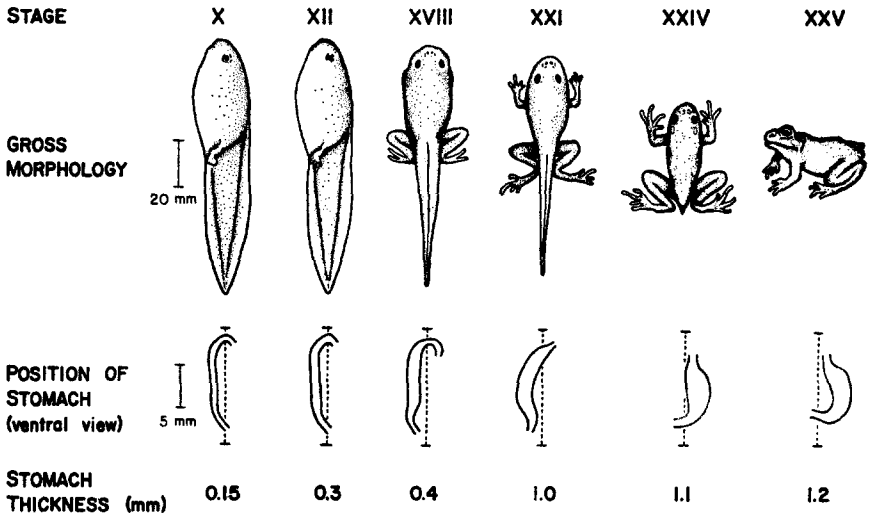

FIGURE 1. Stages of bullfrog tadpole metamorphosis.

bathing solutions and connected either to a high input impedence electrometer (Beckman research model pH meter) or to a Sargent potentiometric recorder (model MR).

For measurement of short-circuit current the stomach was slit along the lesser curvature and the resulting sheet of tissue was mounted between Lucite chambers as previously described (10). The total exposed surface area of the tadpole stomach was 0.32 cm^2. Both sides of the stomach were bathed with HCO_3^--buffered Ringer (4 ml on each side), aerated with 95 % O_2–5 % CO_2. Details for the arrangement of

TABLE I

COMPOSITION OF VARIOUS BATHING SOLUTIONS

Compounds	Ringer solution	Unbuffered secretory solution	Cl^--free Ringer	Choline Ringer
	mM	*mM*	*mM*	*mM*
NaCl	85.5	95.1	—	—
KCl	3.4	—	—	3.4
KH_2PO_4	0.4	—	0.4	0.8
$MgSO_4$	0.9	—	0.9	0.9
$NaHCO_3$	17.6	—	17.6	—
$CaCl_2$	1.8	—	—	1.8
Na isethionate*	—	21.1	85.5	—
K_2SO_4	—	2.5	1.7	—
$CaSO_4$	—	—	1.8	—
Choline Cl^-	—	—	—	84.2
Choline HCO_3^-	—	—	—	17.6
Glucose	11.0	—	11.0	11.0

* Na isethionate ($HOCH_2CH_2SO_3Na$) from Eastman Chemicals was recrystallized two times.

the PD and current-passing bridges, as well as a correction for solution resistance between the PD bridges, have previously been described (10, 11).

Isotopic Flux Measurements Unidirectional fluxes of both ^{36}Cl and ^{22}Na were measured in the tubular stomachs maintained on open circuit, whereas only ^{36}Cl

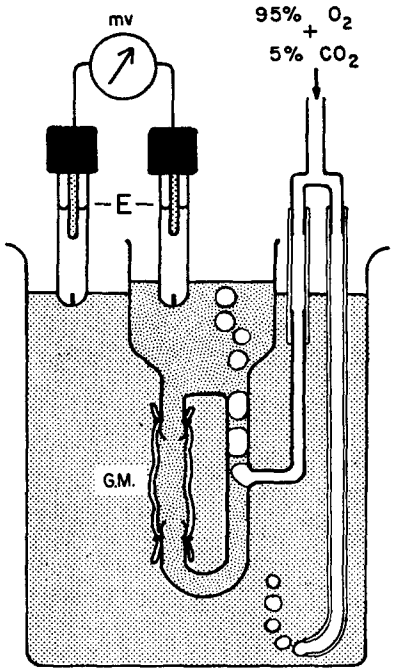

FIGURE 2. Schematic diagram of apparatus used to measure PD and H^+ secretion in bullfrog tadpole stomach. The entire stomach (*G.M.*) was mounted and tied onto appropriate apertures of the glass, circulating chamber. Appropriate solutions were placed inside the chamber (secretory solution) and into the 50 ml beaker in which it was immersed (nutrient solution). A gas phase containing 95% O_2 and 5% CO_2 was used for aeration, circulation, and stirring. A pair of matched calomel electrodes (*E*), placed into the respective bathing solutions, was used for monitoring the PD. When H^+ secretion was measured a glass electrode (not shown) was placed into the secretory chamber to monitor pH.

fluxes were measured in the short-circuited preparations. For an individual experiment the isotope was placed on the predesignated side and after an equilibration of 30–60 min the experimental periods commenced. The solution opposite to that in which the isotope was added originally was removed, the chamber rinsed quickly, and replaced with an equal amount of the initial solution. ^{36}Cl was assayed by liquid scintillation counting (BBOT, toluene, ethoxyethanol counting solution) and ^{22}Na was measured using a gamma-well scintillation counter. Flux calculations have previously been described (12).

Measurement of H^+ Production When H^+ secretion was to be studied, unbuffered saline solution was used on the secretory side. When H^+ secretion occurred, as in-

dicated by a lowering of the pH, standardized 0.02 N NaOH was added by means of a microburette so that the pH of the secretory solution was maintained at a desired end point (pH 5.0). The rate of addition of NaOH is equivalent to the rate of H^+ secretion.

RESULTS

A transmucosal PD was observed in tadpole stomachs at all developmental stages (Table II). Absolute values of the transmucosal PD were quite variable (range −5 to −50 mv), but the polarity was always the same as that observed for adult gastric mucosa; i.e., secretory side negative with respect to the nutrient side. With the exception of stage XXII there was no apparent correlation between the magnitude of the PD and a given morphogenetic stage. Variations may well have been due to problems incurred in the dissection and

TABLE II

TRANSMURAL PD OF STOMACHS ISOLATED FROM TADPOLES IN VARIOUS METAMORPHIC STAGES

Stage	Mean PD	Range	No. of animals
	mv	*mv*	
XI	27.2	16–38	4
XII	28.7	12–38	4
XIII	26.3	24–30	3
XIV	29.0	25–32	3
XV	28.5	27–30	2
XVI	28.0	25–31	2
XVII	28.3	15–39	12
XVIII	27.8	12–43	13
XIX	28.1	13–45	9
XX	22.8	10–30	5
XXI	26.3	9–46	6
XXII	8.0	5–11	5
XXIII	20.0	8–30	5
XXIV	25.5	8–50	11

For all values of the PD the secretory side was negative with respect to the nutrient side.

handling of the tissue during preliminary steps of preparation. Such problems were especially acute in stage XXII of metamorphosis in which the stomach appeared quite constricted, possibly as a result of extensive development of the smooth muscle coats about this stage.

Prior to stage XXIV (or late XXIII) of metamorphosis, tadpole stomachs did not secrete H^+, either spontaneously or in response to added secretagogues. Therefore, for simplicity of presentation the experiments generally have been divided into two major groups: those metamorphic stages prior to the appearance of H^+ secretion, including stages XV to XXII; and the later developmental stages (XXIII to XXV) in which measurable secretion of H^+ occurred.

Electrical and Transport Characteristics of Tadpole Stomach Prior to Development of H^+ Secretion Tadpole stomachs generally appear to be little affected by anoxia in the early stages of development. This is in contrast to the marked sensitivity of the electrical and transport characteristics of the adult (13, 14, 11), or of later metamorphic stages in which the capacity to secrete H^+ was manifest. A comparison of the effects of anaerobiosis during various stages of tadpole development is shown in Fig. 3. The increased sensitivity to anoxia may readily be seen as metamorphosis progresses.

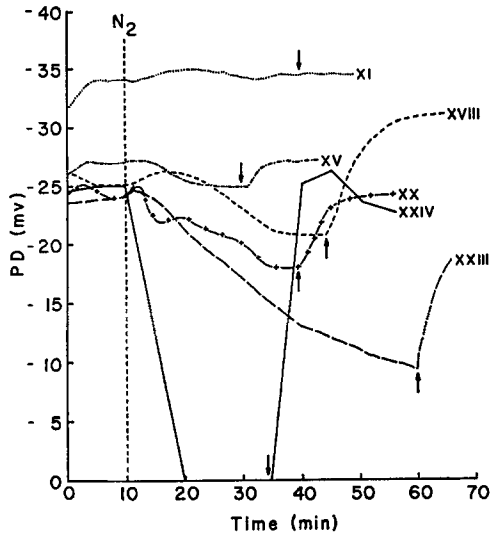

FIGURE 3. A composite of several experiments demonstrating the effect of anoxia on the transmucosal PD of tadpole stomachs at various metamorphic stages. The records have been arranged so that the time coincides for O_2 removal from the solutions bathing each preparation (N_2). At the respective times indicated by the arrows 95% O_2-5% CO_2 was reintroduced into the chambers. For all cases the secretory side was negative with respect to the nutrient side. For a description of the metamorphic stages see Fig. 1 and text.

In contrast to the relative independence of PD with respect to oxidative metabolism in the undeveloped tadpoles, it appeared that glycolytic energy was essential for all metamorphic stages. A typical recording of the transmucosal PD showing the effects of anoxia and of agents reported to inhibit glycolysis is reproduced in Fig. 4. Addition of iodoacetamide or NaF always reduced the PD to zero; a transient increase or "spike," as shown in Fig. 4, was typically observed immediately after addition of glycolytic inhibitors.

Another interesting characteristic of the transmucosal PD in the early metamorphic stages is its sensitivity to cardiac glycosides. On addition of 10^{-3} M ouabain to the nutrient bathing solution (but not to the secretory side) the potential rapidly falls, not to zero, but to about -4 or -5 mv as exempli-

fied by the experimental record shown in Fig. 5. Similar effects were produced with even more dilute concentrations of ouabain (10^{-5} or 10^{-6} M) but the time course of the response was somewhat slower.

A group of experiments was designed to assess the role of interfacial ion gradients in determining the transmucosal PD. When the secretory side was taken first, we found that variations in concentration of Na^+ or K^+ were without effect on the magnitude of the PD. In these experiments Cl^- was maintained constant; K^+ was varied from 0 to 20 mM by substituting KCl for NaCl; Na^+ was varied over a wide range by partial or complete substitution with choline, K^+, Mg^{++}, or Tris.

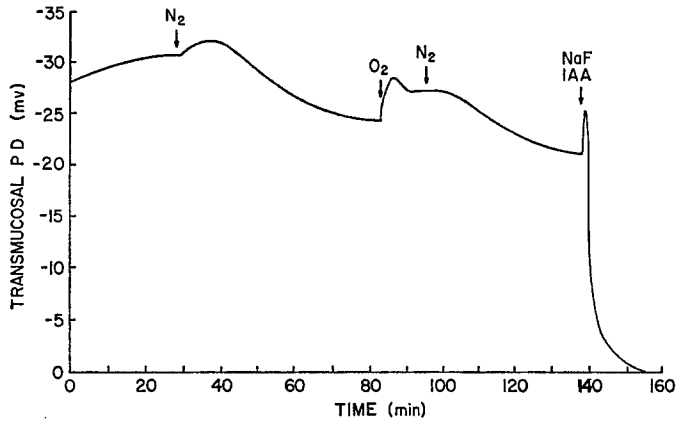

FIGURE 4. Effect of removal of O_2 and the addition of inhibitors of glycolysis on the transmucosal PD of stage XV bullfrog tadpole stomach. The removal of O_2 was associated with relatively small changes in the potential difference whereas the addition of 10^{-3} M NaF plus 10^{-3} M iodoacetamide to the nutrient solution brought about a rapid deterioration of the PD.

Unlike Na^+ and K^+, the concentration of Cl^- in the secretory solution does influence the transmucosal PD. In these experiments Cl^- was replaced by isethionate ($HOCH_2CH_2SO_3^-$). It may be seen from Fig. 6 *b* that a linear relationship existed between the PD and the log [Cl^-] in the secretory solution with a slope of about 29 mv for a 10-fold change in concentration, or about half of that predicted for an ideal Cl^- electrode by the Nernst equation. It is also clear that there was no difference in the observed effect whether [K^+] was maintained constant or whether [Cl^-] was varied at a constant product of [Cl^-] × [K^+] = 500. For our constant product experiments [Cl^-] was decreased by equivalent replacement with isethionate. [K^+] was increased by additions of 0.5 M K_2SO_4 to the bathing solutions. Essentially similar results were obtained if osmolarity of the solutions was maintained constant by an equivalent replacement of K^+ for Na^+.

The changes in transmucosal PD resulting from alterations in [Cl^-], [K^+],

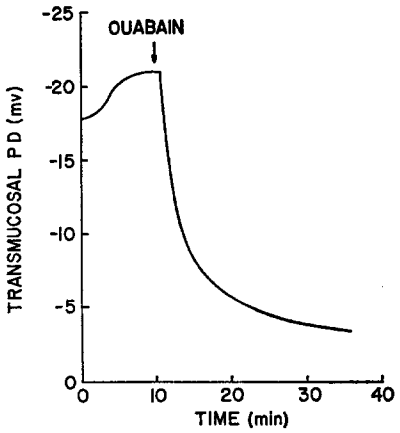

FIGURE 5. Effect of ouabain on the transmucosal PD of stage XVIII bullfrog tadpole stomach. At the indicated time 10^{-3} M ouabain was added to the nutrient bathing solution.

and $[Na^+]$ in the solution bathing the nutrient surface of the tadpole stomach were qualitatively similar to those observed for adult frog gastric mucosa (15–18). The PD varied directly with the log $[Cl^-]$ in the nutrient solution and when the cation concentration was kept constant (isethionate replacing Cl^-), an average slope of 29 mv was observed for a 10-fold change in $[Cl^-]$ (Fig.

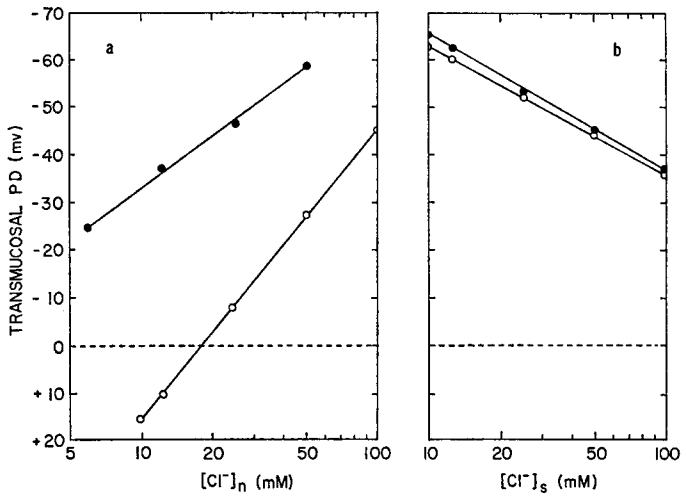

FIGURE 6 *a* and *b*. Typical experiments demonstrating the transmucosal PD in relation to the concentration of Cl^- on the nutrient, $[Cl^-]_n$, or secretory, $[Cl^-]_s$, side of tadpole gastric mucosa prior to the development of H^+ secretion. Variations in $[Cl^-]_n$ when K^+ was held constant at 5 mM (solid circles) produced a change in transmucosal PD with a slope of 29 ± 1.9 mv (SEM, $n = 5$) for a 10-fold concentration change. However, when $[Cl^-]_n$ was varied at a constant product of $[K^+]_n \times [Cl^-]_n = 500$ (open circles), an average slope of 56.6 ± 3.4 ($n = 5$) was obtained for a 10-fold concentration change. Variations in $[Cl^-]_s$ gave essentially the same results whether K^+ was held constant (solid circles) or varied at a constant product of $[K^+]_s \times [Cl^-]_s$ (open circles), that is, about a 30 mv slope for a 10-fold concentration change.

6 *a*). However, when variations in the nutrient solution were made at a constant product of $[K^+] \times [Cl^-] = 500$, about a 60 mv change in PD was observed for a 10-fold change in $[Cl^-]$. This suggested a relatively high permeability of the nutrient interface to both K^+ and Cl^- (19, 16).

As $[K^+]$ of the nutrient solution was independently increased from 5 to 50 mM the decrease in transmucosal PD was qualitatively consistent with a K^+ diffusion potential at that interface (Fig. 7). However, the PD also decreased when the K^+ was reduced below 2–3 mM. Several workers have shown that the PD of adult gastric mucosa decreases by about 30 mv per 10-fold increase in $[K^+]$ of the nutrient fluid (16, 20), but the PD is also reduced, and eventually

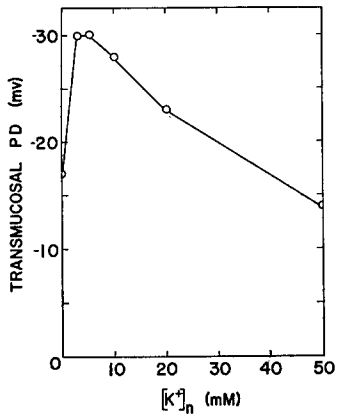

FIGURE 7. Transmucosal PD as a function of the concentration of K^+ in the nutrient bathing medium ($[K^+]_n$).

abolished, as $[K^+]$ is decreased below 1 mM (17). The results for both the presecreting tadpole stomach and the adult mucosa suggest that a K^+ gradient at the nutrient interface contributes to the transmucosal PD. The fall in PD as the $[K^+]$ in the nutrient fluid is reduced to very low values could reflect a loss of K^+ from a critical cellular compartment, perhaps in exchange for Na^+, or it might be the result of a direct effect of K^+ on an electrogenic pump at the nutrient interface. Davis et al. observed no significant decrease in total tissue K^+ when adult bullfrog mucosae were transferred to K^+-free solutions, even though H^+ secretion was abolished and the PD was reduced (17). They postulated a cytoplasmic compartment, relatively low in $[K^+]$, which is essential for maintaining transport and electrical function of the tissue and which is in rapid equilibrium with the K^+ of the nutrient fluid.

Alterations in $[Na^+]$ of the nutrient fluid did not produce a significant change in the tadpole transmucosal PD until concentrations below 15–20 mM were achieved. A typical experiment showing the sequential removal of Na^+ from the nutrient bathing solution is shown in Fig. 8. These results are comparable to the observations of Sachs, Shoemaker, and Hirschowitz who found that the PD and H^+ secretory rate were reduced when the nutrient side of adult gastric mucosa was bathed with Na^+-free solutions (18).

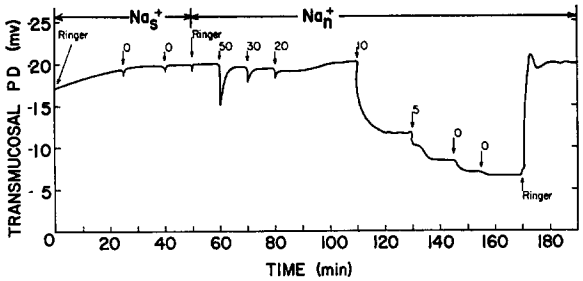

FIGURE 8. The dependence of the PD on Na^+ concentration of nutrient and secretory bathing solutions for stage XVIII tadpole stomachs. During the period indicated by Na^+_s the Ringer solution on the secretory side was replaced and washed with a Na^+-free bathing solution producing no appreciable effect on the PD. The Na^+ concentration in the Ringer solution on the nutrient side was varied during the time indicated by Na^+_n. The numbers adjacent to the arrows indicate the Na^+ concentration of the newly changed bathing solution.

A very interesting change in the cationic-sensitive PD occurred when the tadpole stomach was pretreated with ouabain. As exemplified in Fig. 9, 10^{-4} M ouabain abolished the changes in PD associated with variations of $[K^+]$ on the nutrient side. However, it is also evident from Fig. 9 that no apparent change occurred in the sensitivity to $[Cl^-]$ of the nutrient fluid. The inhibitory effect of ouabain has been demonstrated for a variety of Na^+-K^+ transport systems and its mechanism of action has frequently been attributed to a binding to the enzymic site mediating transport and exchange of these ions. A similar action may take place in tadpole gastric mucosa, but the diminution of an apparently passive K^+ diffusion potential at the nutrient interface was unexpected and must be explained. Two possibilities are that (*a*) ouabain has

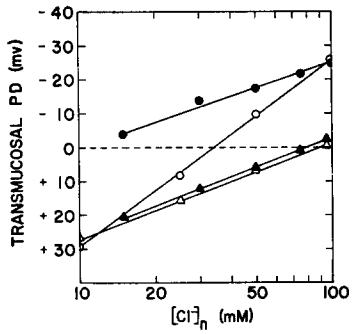

FIGURE 9. The effect of variations in Cl^- concentration on the nutrient side ($[Cl^-]_n$) of a stage XVIII tadpole mucosa before and after the addition of 10^{-3} M ouabain. PD changes prior to the administration of ouabain are indicated by solid circles when K^+ was held constant and open circles when Cl^- was varied at a constant product of $[K^+] \times [Cl^-] = 500$. After the addition of 10^{-3} M ouabain to the nutrient bathing solution, variations in $[Cl]_n$ showed essentially the same effect on PD whether K^+ was maintained constant (solid triangles) or varied at a constant product of $[K^+] \times [Cl^-]$ (open triangles).

an additional effect on the free diffusional characteristics of K^+ through pores or channels in the nutrient membrane, or (*b*) the observed K^+ "diffusion potential" is the result of two boundary potentials (Donnan type) reflecting the association of K^+ with a carrier or binding site within the cell membrane. Ouabain might interact with such a carrier to alter, diminish, or abolish the boundary potentials. Yet another explanation may be based on Rehm's equivalent circuit proposal of a "metabolically" sensitive resistance change in the secretory facing membrane (21).

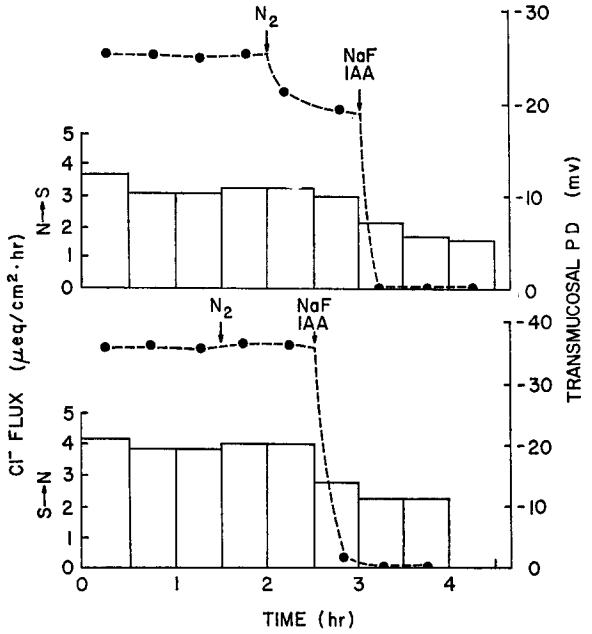

FIGURE 10. The effect of anoxia and glycolytic inhibitors on the unidirectional fluxes o Cl^- across stage XVIII tadpole stomachs. This represents a typical experiment measuring Cl^- flux from nutrient to secretory solution ($N \rightarrow S$) and secretory to nutrient ($S \rightarrow N$) after O_2 had been removed from the bathing solutions (N_2) and after the addition of 10^{-3} M NaF and iodoacetamide to the nutrient side. PD is also shown (solid circles).

In view of the observed asymmetry of the ion-dependent PD changes, it was of interest to measure ion flux across the tadpole stomachs. Our initial flux experiments were performed with stomachs mounted onto the glass chambers and maintained on open circuit. Typical experiments showing the unidirectional Cl^- flux measurements under normal conditions and after inhibition of oxidative metabolism and glycolysis are shown in Fig. 10. Again it may be seen that the PD and the unidirectional fluxes are both relatively independent of oxidative metabolism. Addition of 1 mM iodoacetamide plus 1 mM NaF abolished the PD and reduced both unidirectional fluxes of Cl^- to about one-half of that measured under normal conditions.

A summary of unidirectional flux values for Na^+ and Cl^- is given in Table III. It appears that there is a small net flux of both Na^+ and Cl^- from nutrient to secretory solution, although the significance of net Cl^- movement could not be established by a *t*-test ($P > 0.1$). It may well be that there is a small net movement of Cl^- (see also Table IV) to balance the charge requirements for Na^+ movement, but the unidirectional Cl^- flux is so large relative to Na^+ flux that a definitive statement based on the data in Table III is not possible. However, it is useful to compare the unidirectional flux ratio with the transmucosal PD for both ionic species. Under the conditions used in these experiments (equal concentrations of ions in both solutions at 25°C) the equation

TABLE III

UNIDIRECTIONAL FLUXES OF Na^+ AND Cl^- ACROSS OPEN-CIRCUITED TADPOLE STOMACHS PRIOR TO THE DEVELOPMENT OF H^+ SECRETION*

Ion	Flux direction	Flux	Flux ratio J_{ns}/J_{sn}	Average PD	No. of mucosa	No. of samples
		μeq/cm² per hr		*mM*		
Na^+	J_{ns}	0.30±0.07	2.7	25±0.8	4	16
	J_{sn}	0.11±0.02		27±0.9	5	20
Cl^-	J_{ns}	5.2±0.3	1.05	27±0.9	8	32
	J_{sn}	5.0±0.4		26±1.4	8	32

* The data reported in this table are from stages XVII or XVIII tadpole stomachs.

developed by Ussing (22) and Teorell (23) describing the relationship between the ionic flux ratio and electrochemical potential difference may be simplified as follows:

$$\log \frac{J_{ns}}{J_{sn}} = \frac{zE_m}{0.058} \tag{1}$$

where J_{ns} and J_{sn} are the unidirectional ionic fluxes from nutrient to secretory and secretory to nutrient solution, respectively; z is the charge; and E_m is the transmucosal PD in volts with the nutrient side as reference. The average flux ratio (J_{ns}/J_{sn}) for Na^+ in these experiments was 2.7 which agrees well with the flux ratio of 2.8 calculated from the average PD using equation (1). However, the observed flux ratio for Cl^- is near unity (1.05), demonstrating a marked discrepancy with the calculated flux ratio of 0.34. Thus these results suggest that the asymmetry of transmucosal Na^+ fluxes may be accounted for on the basis of the electrical potential but that Cl^- movement is likely to be coupled with an active process.

Due to inherent practical and theoretical problems in the evaluation of an active transport process solely on the basis of the flux ratio equation, e.g. the nature and magnitude of exchange diffusion processes (22, 23), unidirectional fluxes of Cl^- were measured under conditions of zero transmucosal potential using the short-circuit technique of Ussing and Zerahn (24). These values are reported in Table IV where it is immediately apparent that the bulk of the short-circuit current can be accounted for by net Cl^- flux across the stomach from nutrient to secretory solution. Thus the prediction of an active component of Cl^- flux based on the flux ratio analysis is further supported by the measurements under short-circuit conditions.

TABLE IV

Cl^- FLUX ACROSS OPEN- AND SHORT-CIRCUITED TADPOLE STOMACH PRIOR TO THE DEVELOPMENT OF H^+ SECRETION

Flux direction (No. of mucosae)	Open-circuit		Short-circuit		
	Flux	Mean PD	Flux	Short-circuit current	Mucosal conductance
	μeq/cm² per hr	*mv*	*μeq/cm² per hr*	*μeq/cm² per hr*	*mmhos/cm²*
J_{ns} (6)	6.7±0.7	17.5±2.6	8.1±0.7	2.7±0.4	4.0±0.3
J_{sn} (6)	5.8±0.5	16.7±2.2	5.5±0.5	2.6±0.4	4.3±0.4

Net flux under short-circuit conditions = 2.6 μeq/cm^2 per hr
Mean short-circuit current = 2.65 μeq/cm^2 per hr

All values are given as the mean ± SEM.

It is also significant that the partial ionic conductance of Cl^-, calculated from the backflux of Cl^- (J_{sn}) on the basis of the formulation derived by Hodgkin (25), is larger than the total electrical conductance of the tissue in five of the six stomachs tested (Table IV). A similar observation was clearly pointed out for the adult bullfrog gastric mucosa by Hogben (26) who subsequently suggested a carrier-mediated exchange diffusion for Cl^-. The results shown in Fig. 11 represent additional evidence consistent with a mechanism of Cl^- exchange diffusion in the tadpole stomach. Removal of Cl^- from the trans-side resulted in a prompt reduction of the unidirectional fluxes. Similar evidence has been used to support a mechanism of exchange diffusion in the adult (27, 12); however, caution must be exercised in the interpretation of the present results since the change in PD when Cl^- was removed from either side might account for a large part, if not all, of the transconcentration-dependent Cl^- flux.

The Development of the H^+ Secretory Mechanism It was mentioned earlier that stomachs isolated from bullfrog tadpoles did not demonstrate the capacity to secrete H^+ until relatively late in metamorphic development. The results of

several experiments in which the rate of H^+ secretion was measured first spontaneously, and then in response to secretagogues, are given in Table V for the later stages of tadpole metamorphosis. All tadpoles of stages XXIV and XXV produced measurable rates of H^+ secretion. Except for one of the five tadpoles identified as stage XXIII, and in none of those from earlier stages, was there a demonstrable rate of H^+ secretion.

A typical experiment illustrating some of the electrical and secretory characteristics of the stage XXIV tadpole stomach is shown in Fig. 12; in

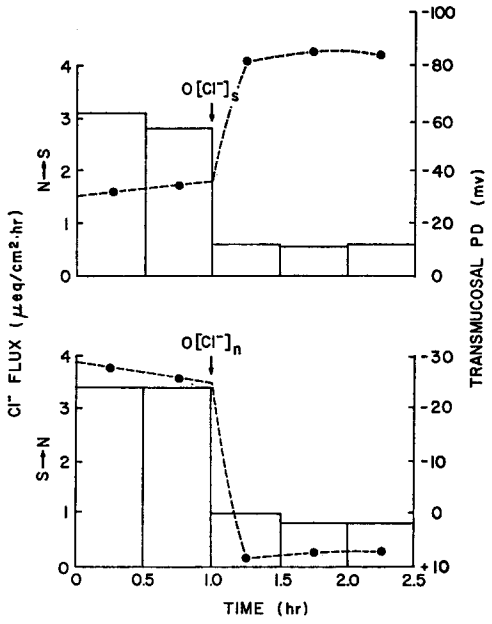

FIGURE 11. Unidirectional fluxes of Cl^- across stage XVIII tadpole stomachs and the effect of the removal of Cl^- from the solution bathing the trans-side. At the time indicated by 0 $[Cl^-]_s$ and 0 $[Cl^-]_n$ the secretory and nutrient solutions, respectively, were replaced by Cl^--free bathing solutions. The transmucosal PD is indicated by the solid circles and dashed line.

many respects they resemble those of the adult gastric mucosa. Pentagastrin stimulated H^+ secretion and generated a small decrease in PD typically seen with the onset of secretion (13). Both the PD and the rate of H^+ secretion are reversibly reduced by anoxia (13, 11), whereas NaSCN characteristically depressed H^+ secretion while elevating the PD (13, 11). Removal of Cl^- from the Ringer solution resulted in a reversal of the PD and a reduction, but not cessation of H^+ secretion (28, 15). Under these conditions both the PD and H^+ secretion are sensitive to anoxia (15). Finally, the incremental addition of Cl^- to the nutrient bathing solution restored the PD and secretory rate towards control values (15).

DISCUSSION

The results presented here clearly show that the secretion of H^+ by the bullfrog tadpole stomach does not begin until very late in metamorphic develop-

TABLE V

ACID SECRETION BY TADPOLE STOMACHS DURING VARIOUS STAGES OF METAMORPHOSIS

Stage of metamorphosis*	XX	XXII	XXIII	XXIV	XXV
Total stomachs tested in chamber	4	5	5	7	4
Stomachs showing spontaneous H^+ secretion	0	0	0	3	2
Stomachs showing H^+ secretion after being stimulated‡	0	0	1	7	4
Mean secretory rate of all responsive stomachs, *μeq/cm² per hr* (± SE)	0	0	0.7	2.0±0.1	2.5±0.4

* Secretory rate for isolated adult bullfrog gastric mucosa ranges from 3 to 5 μeq/cm² per hr.
‡ Either 10^{-4} M histamine·HCl or 10^{-6} M pentagastrin was used as the stimulating agent.

ment (stage XXIII or XXIV). Once the H^+ secretory capacity is established the tadpole stomach appears to be similar to adult gastric mucosa in many of the electrical and secretory characteristics. The commencement of H^+ secretion correlates well with recent studies from this laboratory on the histological

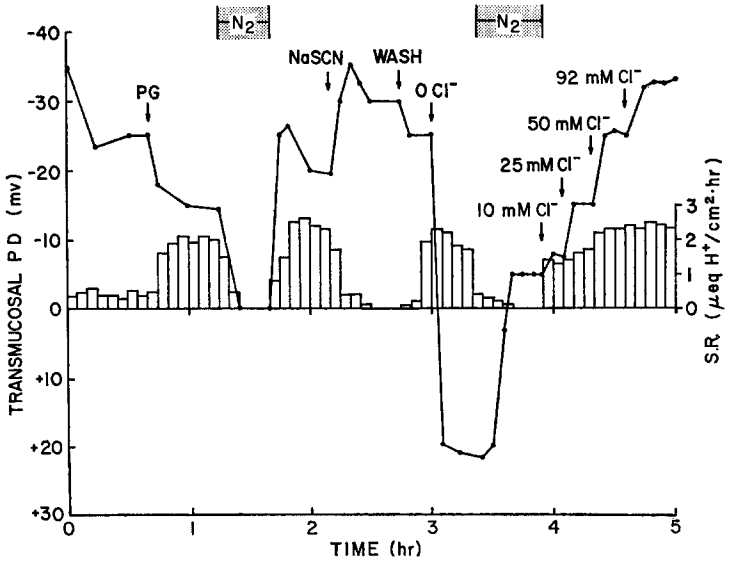

FIGURE 12. Demonstration of the electrical and secretory characteristics of stage XXIV bullfrog tadpole stomach under the influence of various agents or conditions. The introduction of 10^{-6} M pentagastrin (*PG*) stimulated H^+ secretion. Both the PD and H^+ secretion are reversibly reduced to zero by anoxia (N_2) whereas 10^{-2} M NaSCN characteristically depressed secretion while the PD increased. These effects were rapidly reversed by washing. Removal of Cl^- from both bathing solutions (0 Cl^-) resulted in a reversal of the PD and a reduction, but not cessation, of H^+ secretion. Under these conditions both the PD and H^+ secretion are sensitive to anoxia. Finally, the incremental additions of Cl^- to the nutrient bathing solution restored both the secretion and PD to normal values.

and ultrastructural development of the oxyntic cell in bullfrog tadpoles.[1] In mucosae of tadpoles prior to the appearance of H^+ secretion, the developing glandular regions are replete with groups of cells which show a characteristically undifferentiated appearance: large nuclei, many free as well as membrane-bound ribosomal particles, and a general paucity of smooth-surfaced endoplasmic reticulum. In contrast, the glandular cells of stages XXIV and XXV tadpoles showed active and elaborate Golgi structures, numerous tubular elements of the smooth-surfaced endoplasmic reticulum in the apical portion of the cells (possibly derived from synthesis at the Golgi), and patent lumina extending the entire length of the glands. The development of H^+ secretory capacity concomitant with the morphological appearance of the smooth-surfaced endoplasmic reticulum is consistent with the thesis that these membranous elements play a role in the H^+ secretory process (29, 30)[1].

Wright (2) has noted that the isolated fetal rabbit stomach, prior to the development of H^+ secretion, generated a transmucosal PD having the same polarity as that observed in the adult. From his measurements of water and ion movement Wright concluded that net NaCl absorption occurred from the secretory to the nutrient side, with active Na^+ transport very likely representing the driving force, much as is observed for intestinal absorption. A similar absorptive phenomenon does not appear to exist in the case of early tadpole stomachs. From the results with transmucosal ion fluxes it can be concluded that Cl^- is actively secreted, or transported, by the tadpole stomach from the nutrient to the secretory surface. On the other hand, transepithelial Na^+ flux measurements suggest that this ion passively follows the electrochemical potential gradient. These observations at first appear difficult to reconcile with Wright's studies; however, the data for tadpole stomach may be interpreted in a way which would permit an analogy with the mammalian fetal work, and in fact, the analogy may be carried through to characteristics of adult gastric mucosa.

For the early tadpole, the transmucosal PD is dependent upon the presence of Na^+ in the nutrient bathing solution; in addition, the PD is sensitive to ouabain or to the complete omission of K^+ from the nutrient solution. These results are consistent with an hypothesis for a Na^+ transport system in establishing the electrical properties of the developing gastric tissue. A reasonable orientation for such a transport system would be its operation in the direction of cell interior to nutrient side. If the apical secretory membrane of the same cells were relatively impermeable to Na^+, the pump would not provide significant transepithelial transport, but might simply be regarded as a pump-

[1] Forte, G. M., L. Limlomwongse, and J. G. Forte. 1969. The development of intracellular membranes concomitant with the appearance of HCl secretion in oxyntic cells of the metamorphosing bullfrog tadpole. *J. Cell Sci.* 4:709.

leak system at the nutrient interface, analogous to transport systems in many cell membranes, e.g. red cell, muscle, nerve, etc.

Indirect evidence to support a Na^+ pump at the nutrient interface of adult amphibian gastric mucosa may be based upon three lines of inferential evidence (*a*) both PD and H^+ secretion are sensitive to complete removal of Na^+ from the nutrient bathing solution (20); (*b*) ouabain inhibits gastric function (31, 32); and (*c*) Na^+ distribution does not appear to be in electrochemical equilibrium between nutrient solution and epithelial cell interior (33, 34). For mammalian gastric mucosa the evidence supporting a Na^+ pump is more direct. Several groups have demonstrated Na^+ transport from lumen to blood for in vitro mammalian gastric preparations (7, 8). Thus a basic Na^+ transport system may be present in gastric mucosa, and in fact, may be a general characteristic of the entire gastrointestinal epithelium (and all epithelial cells). An overt transepithelial manifestation of the transport system may be a direct function of "passive" properties (relative membrane permeability, histological orientation, etc.). In systems in which net transepithelial Na^+ flux is functionally important, a relatively low resistance to Na^+ movement across the outer facing membrane (or control thereof) is an essential feature (35–38). The apical surface of the small intestinal epithelium has a relatively low resistance to Na^+ movement (38), whereas the apical surface of gastric mucosa has been shown to have a relatively low permeability to Na^+ (39). A possible function for the proposed pump-leak model of Na^+ transport in the gastric epithelium would be to provide specific ion gradients, especially the maintenance of an optimal intracellular $[K^+]$, which appears to be required for normal biochemical (40) and secretory function (41, 17).

The specification of cell types associated with HCl secretion and the nature of the coupling between the transport of H^+ and Cl^- have been recurrent problems in gastric physiology (42–46, 20, 12). Studies with more simplified developing systems, such as the tadpole, may provide a useful experimental approach toward their solution. For instance, the gastric glands of the adult frog stomach contain at least three types of cells: the surface epithelial cell, the mucous neck cell, and the oxyntic cell (47). These cells may be operationally as well as anatomically connected in parallel since tight junctions exist between them. By contrast the early metamorphic stages (X–XV) contain only one epithelial cell type, the columnar epithelial cell, which is a mucous secreting cell and has morphological similarities to the surface epithelial cells of the adult gastric mucosa.[1]

As morphogenesis proceeds, glandular units develop and differentiate in the tadpole gastric mucosa, and eventually the glands become confluent with the surface, resulting in the multicomponent, multifunctional, gastric epithelium of the adult. Several histological studies indicate that the adult epithelium is derived from certain principal epithelial cells of the developing tissue (5, 48,

49). Interestingly, the genetic expression of a basic Cl^- transport and exchange mechanism persists throughout most of tadpole metamorphosis. In the early tadpole stomach Cl^- transport may well be localized to the columnar epithelial cell since it is the only cell type which is present from the very earliest metamorphic stages up through the adult. On the other hand, the physiological expression of the enzymic system associated with H^+ secretion is not manifest until late in the metamorphic development, concomitant with the development of the characteristic oxyntic cell. This great disparity in the expression of the H^+ and Cl^- transport mechanisms lends support to the separate secretory site hypothesis championed by Rehm (42). However, it could still be argued that the expression of the enzymic components associated with H^+ secretion develops as an integral part of, or an adjunct to, the existing Cl^- transport process.

To carry the separate site argument further, one might consider whether H^+ and Cl^- are secreted independently by separate cells, say the oxyntic and surface epithelial cells, respectively. Canosa and Rehm have challenged such an interpretation (46). On the basis that the separate secretory sites were electrogenic they predicted a current flow within the gastric gland, and the absence of a significant voltage drop down the lumen of the canine gastric gland rendered the separate cell version of the theory untenable. However, their conclusion would not obtain if H^+ secretion in the glands were electrically neutral, say as H_2CO_3, and a nonelectrogenic anion exchange occurred at the surface, e.g. HCO_3^- for Cl^-. Although arguments may be offered against this specific mechanism (50), other possible permutations are possible which would permit separate sites without invoking large current flows within the gastric gland.

Thus it would seem likely that H^+, and probably HCl, are secreted by the oxyntic cells of the gastric glands. However, it is not possible to ascertain from the presently available evidence, whether a distinct mechanism for Cl^- transport and exchange persists in the surface epithelial cells of the fully metamorphosed bullfrog gastric mucosa.

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REFERENCES

1. VOLLRATH, L. 1959. Über Entwicklung und Funktion der Belegzellen der Magendrüsen. *Z. Zellforsch.* **50**:36.
2. WRIGHT, G. H. 1962. Net transfer of water, sodium, chloride, and hydrogen ions across the gastric mucosa of the rabbit foetus. *J. Physiol.* (*London*). **16**:281.

3. TONER, P. G. 1965. Development of the acid secretory potential in the chick embryo proventriculus. *J. Anat.* **99**:389.
4. NOMURA, Y. 1966. On the submicroscopic morphogenesis of parietal cell in the gastric gland of the human fetus. *Z. Anat. Entwicklungsgesch.* **125**:316.
5. MENZIES, G. 1958. Observations on the developmental cytology of the fundic region of the rabbits' stomach with particular reference to the peptic cells. *Quart. J. Micr. Sci.* **99**:485.
6. BORNSTEIN, A. M., W. H. DENNIS, and W. S. REHM. 1959. Movement of water, sodium, chloride and hydrogen ions across the resting stomach. *Amer. J. Physiol.* **197**:332.
7. CUMMINS, J. T., and B. E. VAUGHAN. 1965. Ionic relationships of the bioelectric mechanism in isolated rat stomach. *Biochim. Biophys. Acta.* **94**:280.
8. KITAHARA, S., K. R. FOX, and C. A. M. HOGBEN. 1967. Acid secretion and sodium absorption by isolated short-circuited mammalian stomach (cat, dog, rhesus, and human). Abstracts of the Biophysical Society 11th Annual Meeting. Houston, Texas. 49.
9. TAYLOR, A. C., and J. J. KOLLROS. 1946. Stages in the normal development of *Rana pipiens* larvae. *Anat. Rec.* **94**:7.
10. FORTE, J. G., H. HELBOCK, and P. SALTMAN. 1967. A versatile apparatus for membrane transport studies. *Anal. Biochem.* **20**:545.
11. FORTE, J. G. 1968. The effect of inhibitors of HCl secretion on the unidirectional fluxes of chloride across bullfrog gastric mucosa. *Biochim. Biophys. Acta.* **150**:136.
12. FORTE, J. G. 1969. Studies on three components of Cl^- flux across isolated bullfrog gastric mucosa. *Amer. J. Physiol.* **216**:167.
13. CRANE, E. G., R. E. DAVIES, and N. M. LONGMUIR. 1948. Relations between hydrochloric acid secretion and electrical phenomena in frog gastric mucosa. *Biochem. J.* **43**:321.
14. REHM, W. S., W. H. DENNIS, and H. SCHLESINGER. 1955. Electrical resistance of the mammalian stomach. *Amer. J. Physiol.* **181**:451.
15. FORTE, J. G., P. H. ADAMS, and R. E. DAVIES. 1963. Source of the gastric mucosal potential difference. *Nature. (London).* **197**:874.
16. HARRIS, J. B., and I. S. EDELMAN. 1964. Chemical concentration gradients and electrical properties of gastric mucosa. *Amer. J. Physiol.* **206**:769.
17. DAVIS, T. L., J. R. RUTLEDGE, D. C. KEESEE, F. J. BAJANDES, and W. S. REHM. 1965. Acid secretion, potential, and resistance of frog stomach in K^+-free solutions. *Amer. J. Physiol.* **209**:146.
18. SACHS, G., R. L. SHOEMAKER, and B. L. HIRSCHOWITZ. 1966. Effects of sodium removal on acid secretion by the frog gastric mucosa. *Proc. Soc. Exp. Biol. Med.* **123**:47.
19. HODGKIN, A. L., and P. HOROWICZ. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol. (London).* **148**:127.
20. REHM, W. S. 1964. Hydrochloric acid secretion, ion gradients, and the gastric potential. *In* The Cellular Functions of Membrane Transport. J. Hoffman, editor. Prentice-Hall, Inc., Englewood Cliffs, N. J. p. 231.
21. REHM, W. S. 1968. The metabolic state and the response of the potential of frog gastric mucosa to changes in external ion concentrations. *J. Gen. Physiol.* **51**(5, Pt. 2):250.
22. USSING, H. H. 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19**:43.
23. TEORELL, T. 1949. Membrane electrophoresis in relation to bioelectrical polarization effects. *Arch. Sci. Physiol.* **3**:205.
24. USSING, H. H., and K. ZERAHN. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110.
25. HODGKIN, A. L. 1951. The ionic basis of electrical activity in nerve and muscle. *Biol. Rev. (Cambridge).* **26**:339.
26. HOGBEN, C. A. M. 1955. Active transport of Cl^- by isolated frog gastric epithelium. Origin of the gastric mucosal potential. *Amer. J. Physiol.* **180**:641.
27. HEINZ, E., and R. P. DURBIN. 1957. Studies of the chloride transport in the gastric mucosa of the frog. *J. Gen. Physiol.* **41**:101.
28. HEINZ, E., and R. P. DURBIN. 1959. Evidence for an independent hydrogen-ion pump in the stomach. *Biochim. Biophys. Acta.* **31**:246.

29. VIAL, J. D., and H. ORREGO. 1960. Electron microscope observations on the fine structure of parietal cells. *J. Biophys. Biochem. Cytol.* **7**:367.
30. SEDAR, A. W. 1965. Fine structure of the stimulated oxyntic cell. *Fed. Proc.* **24**:1360.
31. DAVENPORT, H. W. 1962. Effect of ouabain on acid secretion and electrolyte content of frog gastric mucosa. *Proc. Soc. Exp. Biol. Med.* **110**:613.
32. MICHELI, L., and R. P. DURBIN. Quoted by D. K. Kasbekar and R. P. Durbin. 1965. *In Biochim. Biophys. Acta.* **105**:472.
33. VILLEGAS, L. 1962. Location of the electrical potential difference in frog gastric mucosa. *Biochim. Biophys. Acta.* **64**:359.
34. DAVENPORT, H. W., and F. ALZAMORA. 1962. Sodium, potassium, chloride and water in frog gastric mucosa. *Amer. J. Physiol.* **202**:711.
35. KOEFOED-JOHNSEN, V., and H. H. USSING. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298.
36. CURRAN, P. F., and J. R. GILL, JR. 1962. The effect of calcium on sodium transport by frog skin. *J. Gen. Physiol.* **45**:635.
37. FRAZIER, H. S., E. F. DEMPSEY, and A. LEAF. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529.
38. SCHULTZ, S. G., P. F. CURRAN, R. A. CHEZ, and R. E. FUIZ. 1967. Alanine and sodium fluxes across mucosal border of rabbit ileum. *J. Gen. Physiol.* **50**:1241.
39. DAVENPORT, H. W., H. A. WARNER, and C. F. CODE. 1964. Functional significance of the gastric mucosal barrier to sodium. *Gastroenterology.* **47**:142.
40. FORTE, J. G., G. M. FORTE, and P. SALTMAN. 1967. K^+-stimulated phosphatase of microsomes from gastric mucosa. *J. Cell. Physiol.* **69**:293.
41. DAVENPORT, H. W. 1963. Sodium space and acid secretion in frog gastric mucosa. *Amer. J. Physiol.* **204**:213.
42. REHM, W. S. 1950. A theory of the formation of HCl by the stomach. *Gastroenterology.* **14**: 401.
43. TEORELL, T. 1947. Electrolyte diffusion in relation to the acidity regulation of the gastric juice. *Gastroenterology.* **9**:425.
44. REHM, W. S., H. SCHLESINGER, and W. S. DENNIS. 1953. Effect of osmotic gradients on water transport, hydrogen ion and chloride ion production in the resting and secreting stomach. *Amer. J. Physiol.* **175**:473.
45. HIRSCHOWITZ, B. I. 1961. Electrolytes in human gastric secretion. *Amer. J. Dig. Dis.* **6**:199.
46. CANOSA, C. A., and W. S. REHM. 1968. Microelectrode studies of dog's gastric mucosa. *Biophys. J.* **8**:415.
47. SEDAR, A. W. 1961. Electron microscopy of the oxyntic cell in the gastric glands of the bullfrog (*Rana catesbeiana*). I. The non-acid–secreting gastric mucosa. *J. Biophys. Biochem. Cytol.* **9**:1.
48. BONNEVILLE, M. A. 1963. Fine structural changes in the intestinal epithelium of the bullfrog during metamorphosis. *J. Cell Biol.* **18**:579.
49. HAYWARD, A. F. 1967. The ultrastructure of developing gastric parietal cells in the fetal rabbit. *J. Anat.* **101**:69.
50. DURBIN, R. P., S. KITAHARA, K. STAHLMAN, and E. HEINZ. 1964. Exchange diffusion of chloride in frog gastric mucosa. *Amer. J. Physiol.* **207**:1177.