Studies of Resting Isolated Frog Gastric Mucosa.* (32066)

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Freshly isolated frog gastric mucosa, mounted as a flat sheet between two chambers, and bathed in appropriate media secretes hydrochloric acid. This occurs in most cases in the absence of any added histamine. Exceptions have been noted for some European species of frogs(1) during certain seasons of the year(2). However, as recently as 1965, Hogben(3) pointed out that "none have so far documented the circumstances that would allow one to reproducibly observe a truly resting non-secreting isolated amphibian gastric mucosa."

A transient cessation of acid secretion and alteration of electrical properties may intervene between the period immediately after mounting the mucosa and the onset of acid secretion. The circumstances responsible for these temporary events are not understood. Variable injuries resulting from the surgical separation of the mucosa may account for these phenomena. The spontaneous secretion of acid which follows is relatively insensitive to added histamine. Also the conditions required for obtaining a non-secreting resting mucosa are presently not known. As a consequence, investigators have used inhibitors of acid secretion as one of the means of comparing physiological and biochemical properties of secreting *versus* non-secreting mucosa. This communication reports studies which have made it possible reproducibly to obtain 'resting' mucosae without the use of any inhibitors and which can be stimulated to almost initial rate of acid secretion by addition of histamine.

Methods and results. Mucosae of *R. catesbeiana,* stripped from their smooth muscle, were mounted between chambers as described by Durbin(4). All stomachs, 2.85 cm^2 in area, were incubated overnight in 14 ml each of histamine-free nutrient solution(4) on the sub-mucosal side and 120 mM NaCl on the luminal side. The nutrient solution was bubbled with a gas mixture containing 95% O_2-5% CO_2 and the secretory solution with 100% O_2. Both the nutrient and NaCl solutions used in overnight studies contained 1 mg/ml each of penicillin and streptomycin. The rates of acid secretion were measured by titrating the unbuffered secretory solution to a constant pH of 6.5 by the pH stat method. The bathing solutions were replaced with fresh solutions at the following times: once within a few minutes of mounting the mucosa, once after reaching initial steady state secretory rate, and then at 3 and 6 hours after the onset of secretion. The following morning, solutions were again replaced 3-4 times at 10-15 minute intervals with fresh solutions without any added antibiotics and histamine. The rates of acid secretion were measured prior to replacement with fresh solutions. The effect of overnight incubations on the secretory rates is shown in Fig. 1. It is evident that the secretory rate, which on an average starts about 6.0 μeq per hour, gradually declines in the absence of added histamine; and at the end of 16 hours is generally very low and of the order of about 0.5 μeq per hour. Omission of glucose from the nutrient solution, used for overnight incubations, gave results similar to those shown in Fig. 1. Glucose was, therefore, excluded to discourage microbial growth during the prolonged overnight incubations, but was present at all other times. Such a slow decline in the rate of acid secretion by frog gastric mucosa in the absence of added histamine has already been indicated from the data of short-term studies of Alonso and Harris(5).

When acid secretion reaches a low rate, after 16 hours of incubation without added histamine, it becomes possible to stimulate acid secretion with exogenous histamine. On

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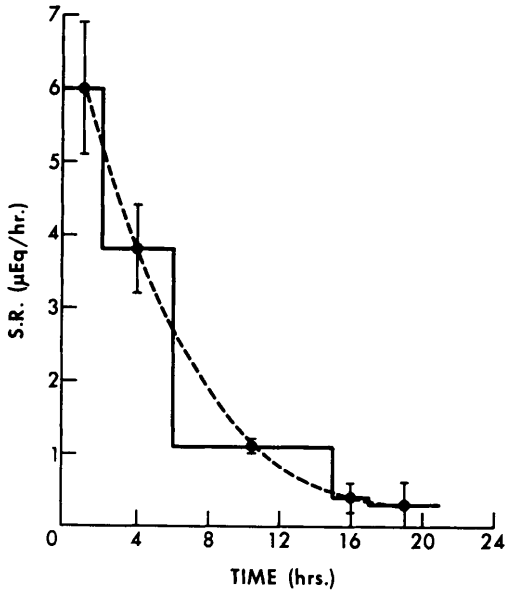

FIG. 1. Effect of omitting histamine from nutrient medium on acid secretion. Average secretory rate for a given period is plotted against time and indicated by solid line. Vertical bars represent ± one S.E.M. for studies with 10 mucosae. Dotted line is drawn by inspection.

addition of histamine to the nutrient solution at a final concentration of 10^{-4} M, which is routinely used in studies with frog gastric mucosa, there is no evidence of an immediate increase in the rate of acid secretion. However, after an average lag period of about 10 minutes (7 to 25 minutes variations), the rate of secretion began to increase (Fig. 2). Maximal secretory rates were reached within about 30-40 minutes of the onset of secretion and maintained for at least 4 hours, at the end of which the experiments were terminated. In some of these studies, the antibiotics were present throughout the experiment, and their presence did not appear to have an effect on the results obtained. Thiocyanate, a known inhibitor of acid secretion, has a similar inhibitory effect on acid secretion by these stimulated mucosae.

Since an average lag period of 10 minutes is observed between addition of histamine and the beginning of an increase in the secretory rate of the preincubated mucosae, it was of interest to determine whether diffusion of histamine was a rate-limiting factor responsible for this lag. The effect of a premature wash-out of added histamine on acid secretion was therefore studied. An interval of 4-6 minutes (which is smaller than the minimum lag period of 7 minutes) was chosen for the exposure of mucosae to 10^{-4} M histamine. After this interval, the histamine-containing nutrient solution was replaced several times at 2 minute intervals with histamine-free nutrient solution. Generally, at least 7 changes were included. The results of this study are shown in Table I. All such

TABLE I. Effect of Premature Removal of Histamine from Nutrient Medium on Gastric Acid Secretion by Resting Mucosae.

Treatment	Secretory rate (μeq/hr)
Fresh mucosae	5.8 (±.4)*
Mucosae, after 15 hr incubation in histamine-free media	0
Preincubated mucosae, as above, exposed to 10^{-4} M histamine for 4-6 min	3.3 (±.5)†

* Figures in parentheses represent ± one S.E.M. for a study with 6 mucosae.
† Maximal secretory rates attained.

mucosae secreted acid although the lag period observed generally increased to about 20 minutes and the rate of secretion was lower than

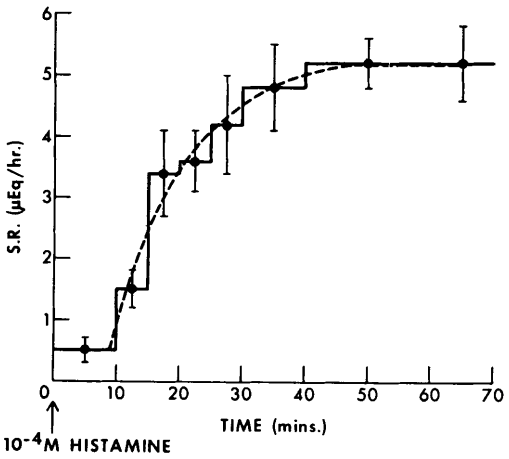

FIG. 2. Time-course of histamine stimulated acid secretion. Gastric mucosae, incubated for 16 hr in absence of histamine, were exposed to nutrient medium containing 10^{-4} M histamine on the serosal side at zero time. No change in secretory rate was observed during approximately the first 10 minutes. After that period, average secretory rates for periods indicated are plotted as a function of time. Vertical bars represent ± one S.E.M. for a study with 6 or more mucosae. Dotted line is drawn by inspection.

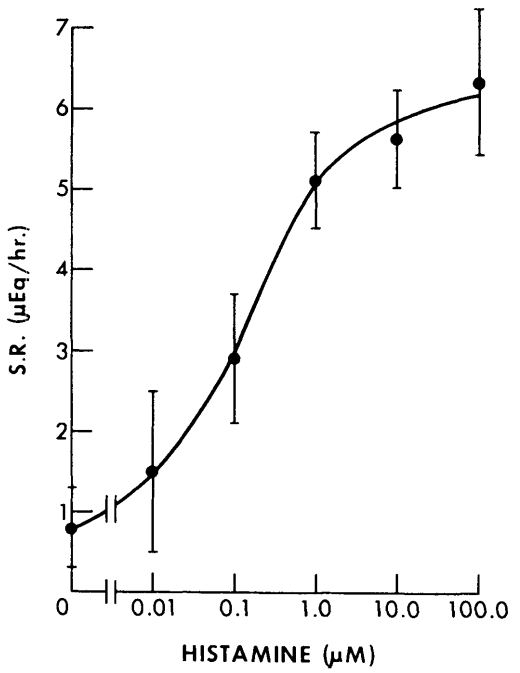

FIG. 3. Effect of various concentrations of histamine on rate of acid secretion. Histamine was added to the resting mucosa in 10-fold increments of concentration. It was held at each concentration for 60 min. except when the concentration was 10^{-8} M (see text). The average steady state secretory rate observed between 40-50 min. following an increase in concentration of histamine is plotted against the appropriate concentration. Vertical bars represent ± one S.E.M. for a study of 6 mucosae.

those of mucosae which were continually exposed to 10^{-4} M histamine.

The preincubated 'resting' mucosae were exposed to histamine ranging in concentrations from 10^{-8} to 10^{-4} M with step-wise 10-fold increments. At 10^{-8} M, responses in terms of acid secretion varied considerably. Some mucosae did not respond even after 90 minutes of exposure; others responded after a lag period of 45-60 minutes. At higher concentrations, the lag periods tended to be considerably smaller. The following procedure was, therefore, adopted for studying the dose-response relationship. When 10^{-8} M histamine was used, the mucosa was assumed to have reached a steady state secretory rate at 90 minutes following addition of histamine. At all other concentrations steady state rates resulted after about 40 minutes. The rate observed was therefore noted. The results of this study are shown in Fig. 3.

Preincubation of the mucosa for 15 hours in the absence of added histamine always resulted in an increase of the open circuit potential difference across the mucosa. The initial p.d. was measured soon after a steady state secretory rate was attained by the freshly mounted mucosa; measurements on 10 such mucosae yielded an average value of — 18 ± 3 mv.‡ The resting mucosae, at 15 hours, had a p.d. of — 29 ± 3 mv. The stimulation of the resting mucosae with 10^{-4} M histamine did not result in a significant change in p.d. and had a value of — 28 ± 2 mv. The corresponding short-circuit current values were 244 ± 29 μamps for fresh mucosae, 165 ± 26 μamps for resting mucosae and 207 ± 25 μamps for the mucosae stimulated from the resting state.

Kasbekar and Durbin(6) reported recently on the isolation of an ATPase from frog gastric mucosa. On the basis of its properties, they suggested that this enzyme may participate in the sequence of reactions leading to acid secretion by the stomach. Many workers have also suggested that ATP may be the immediate source of energy for acid production(7,8). It was of interest, therefore, to compare the activity of this ATPase as well as of ATP levels in the resting and stimulated mucosae. Accordingly, the resting and 10^{-4} M histamine-stimulated mucosae were removed at the appropriate times from the chambers, homogenized and assayed, as described earlier for ATPase activity(6) and for ATP levels(9) in separate experiments. The results of this study are given in Table II. There is little difference between the ATPase activity of resting and stimulated mucosae. These observations are consistent with the previous finding of Kasbekar and Durbin(6) as well as those of Sachs, Mitch and Hirschowitz(10) that histamine at concentrations ranging from 10^{-5} to 10^{-3} M has no significant effect on the isolated gastric "microsomal" ATPase activity. The ATP levels of the stimulated mucosae, on the other hand, showed a small increase over those of the resting stomachs.

Discussion. The studies described indicate

‡ Standard error of mean.

TABLE II. ATPase Activity and ATP Levels of Resting and 10^{-4} M Histamine-Stimulated Mucosae.

	No. of mucosae	Resting mucosae	Stimulated mucosae
ATPase activity $\left(\frac{\mu\text{moles } P_i}{\text{min} \times \text{mg protein}}\right)$	5	.29 (±.08)	.27 (±.07)*
ATP levels (μmoles/g wet wt)	7	.96 (±.02)	1.09 (±.08)

All gastric mucosae were incubated for 15 hr in histamine-free nutrient medium. At the end of this period, one group was treated with 10^{-4} M histamine and the other group was used as a control. One to 2 hours after addition of histamine, when the experimental group had reached steady state secretory rate, the mucosae were removed from the chambers, and used for ATP and ATPase assays. ATP and ATPase levels were determined in separate groups of experiments.

* Figures in parentheses represent ± one S.E.M.

that prolonged incubation of the mucosa in histamine-free nutrient media can result in a resting state. This resting mucosa is sensitive to concentrations of histamine ranging from 10^{-8} to 10^{-4} M. Histamine is known to be present in the frog gastric mucosa(11); it may be responsible for the spontaneous acid secretion, and is perhaps slowly lost during the prolonged incubation. Although the mechanism of histamine loss from the isolated frog stomach is not known at the present time, Code(12) has discussed the various possibilities with respect to mammalian stomachs. Loss may result from destruction in the mucosa or by removal *via* gastric juice.

Since the resting mucosa obtained under these conditions responds to exogenously added histamine, it appears that histamine is directly involved in the sequence of reactions leading to acid secretion. The significance of the lag period observed between exposure to histamine and the onset of increase in the rate of acid secretion is not clear. There are several possible explanations. Diffusion of histamine may be a rate-limiting factor. The wash-out studies point to the possibility that some histamine is taken up and may be trapped in an unknown manner in the mucosa, but is yet unavailable to the secretory apparatus. However, the fact that there is only a partial restoration of secretion indicates that more than one factor may be involved.

Very little is known about the intimate mechanisms by which histamine stimulates gastric acid secretion. A direct effect of histamine on the gastric ATPase, which has been postulated as a likely enzyme involved in the sequence of reactions leading to acid secretion, may be ruled out at this time. Similarly, while the possibility that histamine may act by increasing the ATP levels appears to be unlikely, its effect on ATP turnover remains to be investigated.

Summary. Freshly isolated frog gastric mucosa secretes hydrochloric acid, under appropriate conditions, in the absence of added histamine. However, if the mucosa is incubated with histamine-free media, the acid secretory rate decreases to a very low rate after approximately 16 hours, the p.d. increases, and the short-circuit current declines. Addition of 10^{-4} M histamine to the nutrient medium bathing the serosal side restores acid secretion to about 80% of the initial rate; there is little change in p.d. and an increase in short-circuit current is observed. No difference exists in the ATPase activity of the resting and histamine-stimulated mucosa; the ATP level of the stimulated mucosa, on the other hand, is slightly greater than that of the resting mucosa. The procedure of preincubation of the mucosa in histamine-free media has made it possible for the first time reproducibly to obtain resting mucosae which can be stimulated by added histamine.

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Further Observations on the Inhibitory Action of Ammonium Chloride on Influenza Virus.* (32067)

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Ammonium salts at low concentration inhibit the growth and cytopathic effect of type A influenza viruses(3,10). Other myxoviruses are affected slightly or not at all and the only other species for which similar results have been reported is a member of EMC group(8). Inhibition of viral penetration has been suggested as a common mode of antiviral action between NH_4Cl, adamantanamine, and a piperazine derivative(1,6,7,9). In earlier experiments with a different cell-virus system (3) we had been unable to demonstrate a significant effect of ammonium phosphate on penetration of influenza virus. Also inhibition of a type B virus in chorioallantoic tissue was observed although it has been reported by others that neither adamantanamine nor NH_4Cl are effective against this type of influenza(6). Since an accurate knowledge of the mode of action of effective inhibitors is of fundamental importance it seemed desirable to investigate the reasons for these discrepancies.

Materials and methods. The media and methods used for preparation of roller tube cultures of chorioallantoic tissue have been described(2,4).

Virus. The PR_8 type A strain and the Lee type B strain of influenza virus were propagated in the allantoic sac of chick embryos. The allantoic fluids had hemagglutinin (HA) titers of 1,000 to 5,000 HA units per ml. Virus/cell multiplicities were estimated as follows. For the PR_8 strain one HA unit is approximately 10^6EID_{50}. The roller tubes each contained 40 mg (wet weight) of tissue in 4 ml of medium and a final dilution of virus giving 40 HA units per ml would represent an input multiplicity of 10^6EID_{50}/mg of tissue. Assuming that there are 10^5 cells/mg and that all cells come in contact with virus the resulting EID_{50}/cell would be approximately 10.

Antiserum. Sera from rabbits immunized with type A influenza virus had hemagglutination inhibition (HI) antibody titers between one and five thousand as measured by a standard method. Since these sera contained antibodies to chicken tissue adsorption with 10% erythrocyte suspensions was necessary before they could be used in experiments with chorioallantoic tissue. When antiserum having a titer of 1:5000 *vs* 8 HA units was used at a final dilution of 1:200 this gave 25 HI units per ml or an antibody excess of approximately 10-fold over virus input.

Viral replication can be limited to a single cycle by placing preinfected cells in a medium containing antiviral antibody. The yield of cell-associated virus after a period of replication is then proportional to the amount of virus that has penetrated cells. When antiserum is removed after an hour's contact with the cells, as has been done in other laboratories, multiple growth cycles occur unless all cells are infected initially with a large excess of virus. Treatment of infected cells with antiserum was performed in two different

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