THE CONSEQUENCES OF MUTATION DURING THE GROWTH OF BIOCHEMICAL MUTANTS OF ESCHERICHIA COLI

II. The Inhibition of Histidine-independent Bacteria by Histidineless Bacteria in Unshaken Cultures[1]

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During the growth of a histidineless mutant (h−) of *Escherichia coli* there appear, with a low frequency, histidine-independent (h+) back mutants (Ryan, 1948). These h+ back mutants grow in the absence of histidine at the same rate and to the same extent as do the h− parents when supplied with optimal concentrations of histidine. Unlike their h− parents, these h+ back mutants, when grown by themselves, behave in a way that is independent of the histidine content of the medium. In the presence of optimal concentrations of histidine there is no selective advantage to synthesize this compound. Mixtures of h+ and h− organisms, when grown under such conditions, retain their initial proportion for many generations. In the absence of histidine, on the other hand, there is a selective advantage to synthesize this substance, and h+ bacteria will overgrow the population of h− organisms from which they were derived.

On intermediate, limiting concentrations of histidine h+ bacteria can undergo an amount of growth that is limited by the number of h− bacteria present in the culture (Ryan and Schneider, 1948). As a consequence, adaptation, the overgrowth of an h− culture by h+ organisms produced within it, becomes progressively less as the histidine concentration increases. In some way the h− organisms, when grown on suboptimal concentrations of histidine, can prevent the full growth of h+ bacteria. The mechanism of this action is the subject of the present and the following papers (Ryan and Schneider, 1949*a*,*b*).

EXPERIMENTAL PROCEDURES

The material and most of the methods used in this study have already been described (Ryan and Schneider, 1948). In addition, hydrogen ion concentrations were determined with a Beckmann model H pH meter. The pH of the medium was determined after autoclaving, and modifications were made by the addition of either sodium hydroxide or hydrochloric acid. When the pH of a filtrate, or of a whole culture, was to be raised, a determination of the amount of sterile alkali required was first made on an aliquot. Total additions to the cultures were always less than 10 per cent of the original volume. Sterile filtrates were prepared by passage through pyrex UF sintered glass crucibles. All experiments reported in this paper were run separately at least twice; most of them were run many times.

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All cultures, unless otherwise mentioned, were kept stationary. They were not, strictly speaking, unagitated. Before turbidity determinations were made each culture was spun by hand to make the suspension as homogeneous as possible. The number of turbidity determinations and the amount of spinning was an uncontrolled variable in these experiments. However, the cultures were not continuously shaken as was the case in experiments to be reported later (Ryan and Schneider, 1949*a*).

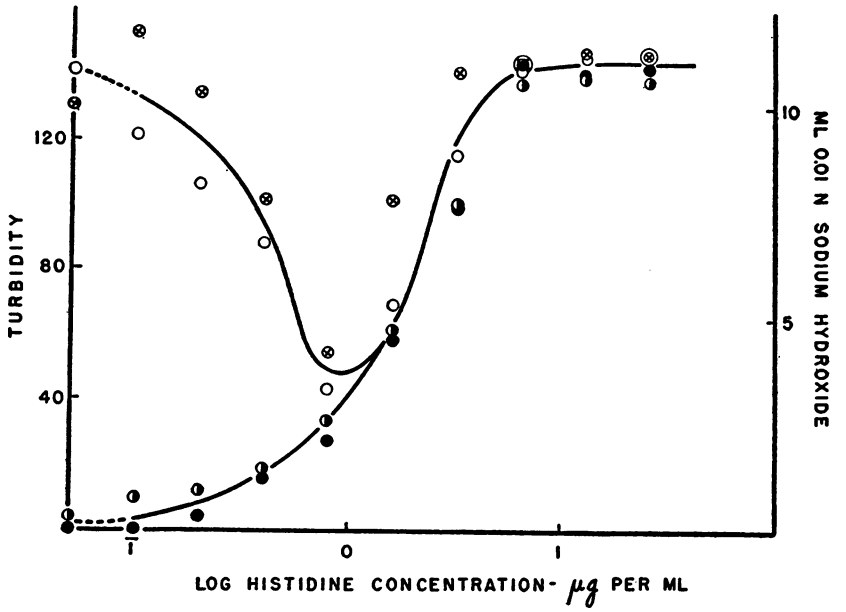

Figure 1. The parallel behavior of growth and acid produced by h− cultures on different concentrations of histidine. The solid circles are for unadapted growth at 14 hours and the half-open circles for the amounts of 0.01 N sodium hydroxide required to bring the cell-free filtrates of the unadapted cultures to a pH of 7.0. The open circles are for adapted growth (38 hours) and the crossed circles for the base equivalents of the acidity of filtrates of the adapted cultures. The dotted lines go to the levels of growth and acidities on minimal medium devoid of histidine.

RESULTS

The limitation of growth. Figure 1 shows the levels of growth attained before and after the adaptation of 19 ml h− cultures in unshaken test tubes. During growth, acid is produced in amounts proportional to the number of cells that are formed; the titratable acidity is shown, in figure 1, to parallel the levels of growth before and after adaptation. As a consequence, the pH of the culture medium is reduced to the varying extents shown in figure 2. Where growth is least after adaptation, there the pH has been reduced to the least extent. The level of adaptive growth is stable and does not change for a period of several weeks, but the pH continues to drop, reaching a limit of about 5.5. Yet these pH's, even shortly after adaptation when they may be as high as 6.5, limit growth on intermediate concentrations of histidine. This has been shown in two ways. In the first place,

when whole h− cultures were allowed to adapt on a medium containing 0.4 or 0.8 μg histidine per ml and several hours later were brought to a pH of 7 (with sterile sodium hydroxide or sterile mixtures of sodium or potassium phosphate), growth was reinitiated, although at a slow rate. During the same period no growth at all was shown by controls that were allowed to remain at the pH produced by adaptation. Similarly, when sterile filtrates of h− cultures, made sometime after adaptation on 0.4 and 0.8 μg histidine per ml, were inoculated with h− or h+ organisms, no growth occurred unless inoculation was preceded by the sterile neutralization of the filtrates. By neither technique could growth be obtained with the addition of histidine, sugar, or any of the mineral constit-

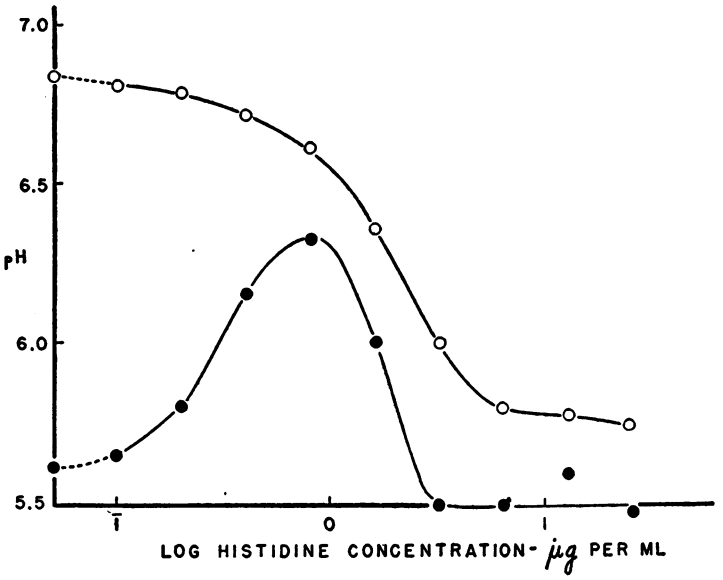

Figure 2. The pH of whole unadapted (14-hour) and adapted (38-hour) h− cultures as a function of histidine concentration. The upper curve is for unadapted and the lower curve for adapted cultures. The dotted lines go to the pH's produced on minimal medium devoid of histidine.

uents of minimal medium singly or in combination, provided that the pH was not raised. It may be concluded that pH limits the poor growth obtained at the dip in the adaptation curve.

Since this is so, an increase in the buffer capacity of the medium should, by resisting the effects of acid production, allow for greater growth. The minimal medium used contains 3 g K_2HPO_4 and 1 g KH_2PO_4 per liter and, after autoclaving, has a pH of about 6.8. Figure 3 shows the effect of increasing the concentration of these phosphates to various extents, yet always maintaining the 3:1 ratio. In the range between 0.4 and 0.8 μg of histidine per ml, where the adaptation curve is depressed, a striking effect is noticed. Considerably more growth is achieved on double and quadruple phosphate medium than on single-strength medium. (Eight- and sixteenfold increases in phosphate appear to be toxic.) Similarly the amount of growth achieved on lower concentrations of histidine or

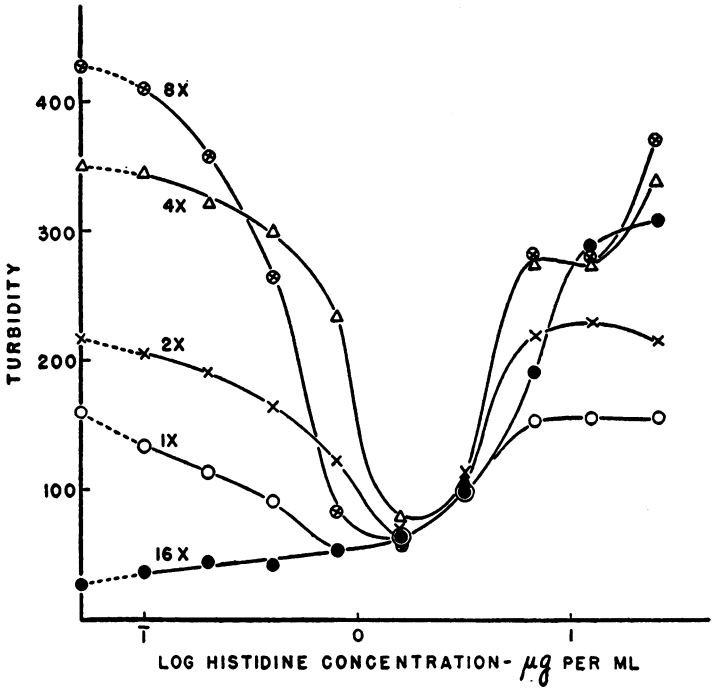

Figure 3. The level of adaptive growth attained by h− cultures after 46 hours on different concentrations of histidine in medium containing different amounts of phosphate. The numbers on the curves refer to the concentrations of phosphate as described in the text. The dotted lines go to the levels of growth on medium devoid of histidine.

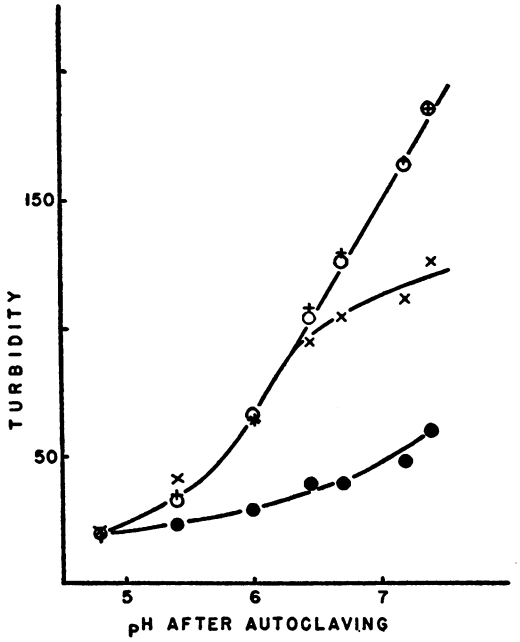

Figure 4. The effect of initial pH (after autoclaving) on the stationary level of preadaptive growth of h+ and h− cultures in the presence of different concentrations of histidine. The open circles enclose points representing the level of growth of h+ bacteria after 16 hours in the presence of 0, 0.4, and 3.2 μg histidine per ml. The solid circles, X's, and +'s refer to the levels of growth attained by h− bacteria in the presence of 0.4, 3.2, and 25 μg histidine per ml, respectively.

in its absence and on optimal concentrations of histidine is greater the larger the buffer capacity of the medium. In the presence of 1.6 and 3.2 μg histidine per ml, however, the amount of growth is independent of the buffer capacity, and on the different media the same amounts of growth reduced the pH to different levels between 6.8 to 6.5. Thus, on low and on optimal concentrations of histidine the amount of buffer, probably through its effect in resisting the decrease in pH, seems to determine the final level of adaptive growth; but on 1.6 and 3.2 μg histidine per ml some other factor is involved.

In order to test this point the amount of growth of h− and h+ cultures was determined on different concentrations of histidine at different initial pH's

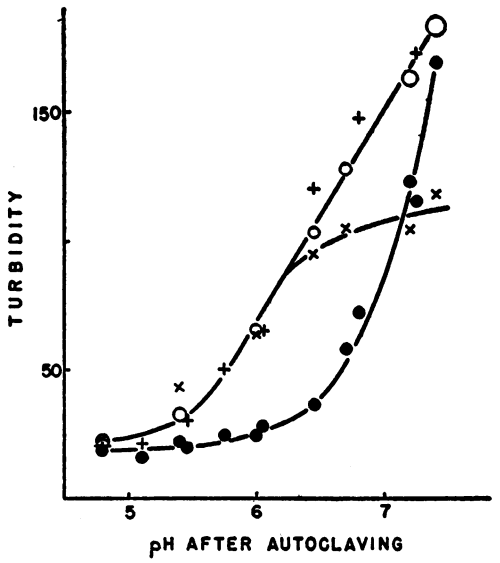

Figure 5. The effect of initial pH (after autoclaving) on the final adaptive level of growth of h+ and h− cultures in the presence of different concentrations of histidine. The open circles enclose points representing the level of growth of h+ bacteria after 38 hours in the presence of 0, 0.4, and 3.2 μg histidine per ml. The solid circles, X's, and +'s refer to the levels of growth attained by h− bacteria in the presence of 0.4, 3.2, and 25 μg histidine per ml, respectively.

(figures 4 and 5). Cultures of h+ bacteria, irrespective of the histidine concentration they contained, showed a regular increase in growth with increase in pH. The same sort of increase was exhibited by h− cultures when grown on an optimal (25 μg per ml) concentration of histidine. Before adaptation, in the presence of 0.4 μg per ml, there was also an increase in growth with a rise in pH, but at any pH the growth was less than that achieved on optimal histidine concentrations. After adaptation there is a sharp increase in the levels of growth above pH 6.5 due to the multiplication of the small number of h+ mutants in the h− culture. Below pH 6.5, however, h+ mutants do not overgrow the culture and adaptation does not occur. Obviously the h+ bacteria are influenced in their behavior by the h− organisms present. In the presence of 3.2 μg histidine adaptation did not occur at any of the pH's studied (4.8 to 7.4) despite the fact that live

h+ bacteria were shown (by plating) to be present. Further, on this concentration the total growth was approximately the same at pH 6.5 and 7. This finding is consistent with the results obtained with media buffered to different extents. But it is still necessary to explain why h+ organisms at an initial pH of 6.5 can grow to an optical turbidity of about 100, although in the presence of h− organisms they are prevented from growing at a pH of 6.5 and adaptation does not occur.

Test of inhibition. This restriction in the growth potential of the h+ organisms could be due to the addition to, or the removal from, the medium of something by the h− bacteria. If the latter were true, then growth should be reinitiated by the addition of fresh medium (unless, of course, the restriction involved a permanent change). A series of h− cultures were allowed to adapt on histidine concentrations from 0 to 25 μg per ml. At this time solutions of salts, sugar, and histidine, singly and in combination, were brought to the pH of the adapted cultures and added to them. The volumes added were one-tenth of the volumes of the cultures, and the amounts of solute were ten times that in fresh medium containing 25 μg histidine per ml. Despite this, no new growth ensued. The limitation of growth evidently was not due to the removal of something from the medium.

Consequently something that we can call an inhibitor or inhibitors must have been added by the h− bacteria. There are two simple hypotheses regarding the production of an inhibitor by h− bacteria that would explain the dip in the adaptation curve. The first assumes that h− bacteria produce an inhibitor only on limiting concentrations of histidine. In this event filtrates from cultures grown on such concentrations should be the least favorable for new growth. The second hypothesis assumes that the h− bacteria produce an inhibitor in proportion to their number and that this inhibitor is specific against h+ bacteria. In this event filtrates from cultures grown on optimal concentrations of histidine should be the least favorable. In order to discriminate between these hypotheses sterile filtrates were prepared of h− cultures allowed to adapt on different histidine concentrations. These were neutralized; aliquots were kept in this form or were supplemented with optimal amounts of histidine and were then inoculated with h+ or h− bacteria. This experiment was performed four times and the results were never the same. All experiments, however, had some features in common. First, unneutralized control filtrates supported no growth after inoculation. Second, filtrates from some histidine concentrations supported the growth of fewer bacteria than others. The most unfavorable filtrates, however, came, in one experiment, from cultures grown on 0.4 μg histidine per ml and, in another, from cultures grown on 25.6 μg per ml. Further, when the adapted filtrates were neutralized and mixed with equal volumes of double-strength fresh medium with or without histidine, results of the same sort were obtained. Some of the filtrate experiments performed seemed consistent with the first hypothesis, but the remainder were inconsistent with it. The remainder were also inconsistent with the second hypothesis because filtrates from cultures that supported the growth of large numbers of h− organisms were equally unfavorable for the growth of h−

and h+ bacteria. There was no evidence that the filtrates contained inhibitors specific against h+ organisms.

In order to test the inhibition hypothesis further, filtrates were assayed for their effect on the rate of growth of cultures maintained in the logarithmic phase by a method described in the following paper (Ryan and Schneider, 1949*a*). The filtrates were neutralized, concentrated under vacuum, and added in volumes not over 10 per cent of the total to cultures of h− and h+ bacteria in the logarithmic phase of growth. Figure 6 shows the effect of adding a filtrate from an

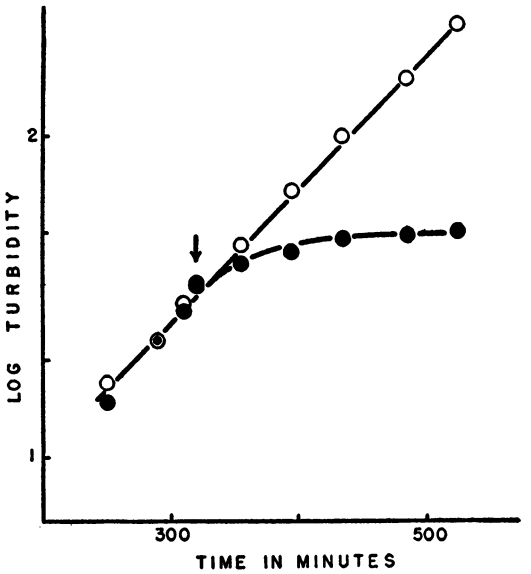

Figure 6. The inhibition of the rate of growth of h+ bacteria by a filtrate of a culture of h− bacteria. To the left are shown the growth curves of two shaken cultures of h+ bacteria in minimal medium. At the time indicated by the arrow one culture received a sterile concentrate of uninoculated minimal medium evaporated from 10 to 1 ml. The upper curve shows the relatively unimpaired growth of the h+ bacteria which followed this addition. The other culture received at the same time a sterile concentrate of a filtrate of an unshaken culture of h− bacteria grown for 19 hours in the presence of 25 μg histidine per ml and evaporated from 10 to 1 ml. The lower curve shows the inhibited growth of the h+ bacteria following this addition.

h− culture to h+ bacteria. The rate of growth was decreased to about 20 per cent of the initial value. On the other hand, the addition of concentrated minimal medium did not appreciably affect the rate of growth. Such behavior on the part of controls was consistent; in eight experiments the rate of growth after the addition of concentrated minimal medium was 106 ±14 per cent of the rate before addition. That the concentration of the filtrate did not in itself produce the inhibitor(s) was shown by experiments in which filtrates from h− cultures grown in the presence of 25 μg histidine per ml were concentrated, then restored to their initial volume. They were compared with aliquots of the same filtrate that had not been concentrated. The two types of preparations in several experiments gave inhibitions that were within 10 per cent of one another. Further, the inhibitory

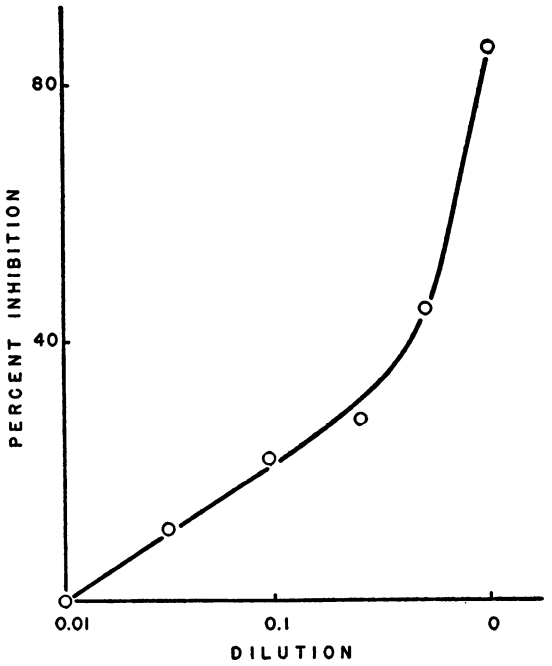

Figure 7. The effect of dilution on the inhibitory action of a filtrate of an h− culture on the rate of growth of h+ bacteria. The h− culture had been grown for 18 hours in the presence of 25 μg histidine per ml; it was then filtered and the filtrate was evaporated from 10 to 1 ml. The h+ culture was grown in shaken minimal medium and the percentage of inhibition was calculated from the change in the logarithmic rate of growth before and after the addition of the filtrates.

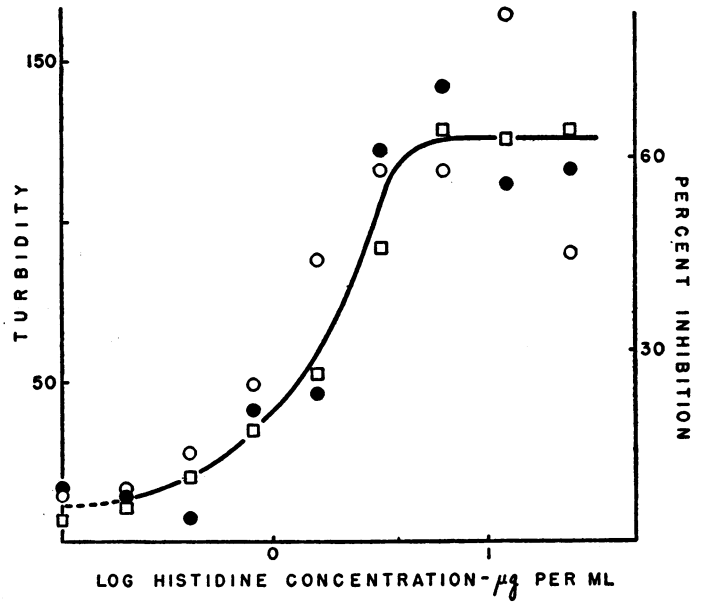

Figure 8. The extent of inhibition, by filtrates of unadapted h− bacteria grown on different concentrations of histidine, of the rate of growth of h− and h+ organisms. The abscissa denotes the histidine concentrations on which the h− bacteria were grown prior to filtration and concentration from 10 to 1 ml. The ordinate on the left measures the turbidity of these cultures. The ordinate on the right measures the percentage of inhibition as gauged by the difference in the logarithmic rate of growth of h+ and h− bacteria before and after the addition of the filtrates. The squares refer to the level of growth of the h− cultures after 19 hours just before filtration, the solid circles to the percentage of inhibition of h− bacteria grown in shaken medium supplemented with 25 μg histidine per ml, and the open circles to the percentage of inhibition of h+ bacteria grown in shaken minimal medium. The dotted line goes to points for filtrates of h− bacteria in the absence of histidine.

activity of the filtrate concentrates decreased upon dilution (figure 7) and would not stand complete desiccation or autoclaving for more than a few minutes.

Figure 8 shows the activities of filtrates of h− cultures grown in the presence of different concentrations of histidine. Although there is considerable scatter among the points, it is evident that the extent of inhibition of growth rate is proportional to the amount of growth that had ensued in the cultures from which the filtrates were prepared. In these experiments the final levels of growth of the cultures to which the filtrates had been added were decreased as much as 20 per cent when the rate of growth was slowest. Similarly, filtrates from adapted cultures showed inhibitions in proportion to the number of bacteria in the cultures from which they were made. Once again these findings are inconsistent with the hypothesis claiming the production of an inhibitor by h− bacteria only on intermediate concentrations of histidine where the dip in the adaptation curve occurs. Further, the filtrates were equally inhibitory to h+ and h− bacteria. Thus the inhibitor (or inhibitors) is not specific against the h+ back mutants and its action cannot explain, at least in a simple fashion, the depression of adaptation on intermediate concentrations of histidine. Indeed, filtrates from cultures of h+ bacteria show the same nonspecific inhibitory activity and to the same extent as filtrates from h− cultures containing similar numbers of bacteria. A later paper in this series will present evidence revealing the way in which the inhibitor (or inhibitors) depresses adaptation (Ryan and Schneider, 1949*b*).

SUMMARY

In unshaken cultures of *Escherichia coli*, in which the pH decreases in proportion to the number of bacteria, the adaptive growth of histidine-independent back mutants takes place until intolerable pH's arise. The pH tolerance of these back mutants is determined by the proportion of parental histidineless bacteria also present in the culture. This restriction by the histidineless bacteria is not brought about by the depletion of something from the medium but rather by the production of some nonspecific substance (or substances) that is formed in proportion to the number of bacteria. These results, in conjunction with those reported in the following paper (Ryan and Schneider, 1949*a*), are discussed in the final paper of this series (Ryan and Schneider, 1949*b*).

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