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

III. The Inhibition of Histidine-Independent Bacteria by Histidineless Bacteria in Aerated Cultures[1]

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The preceding paper in this series (Ryan and Schneider, 1949*a*) demonstrated that the adaptation of unshaken histidineless (h−) cultures is limited by the acid produced during growth. The various pH's developed at intermediate histidine concentrations prevent the further growth of the histidine-independent (h+) mutants produced in these cultures. The sensitivity of the h+ bacteria to the various pH's is conditioned by something produced by h− bacteria during their growth. It was thought that the acid production might be a function of the partially anaerobic metabolism that must occur in the depths of the 10 ml of medium that were contained within the 15-by-150-mm pyrex test tubes (Stokes, 1949). Aeration might prevent this acid production and allow the phenomenon of adaptation to be studied without the complication of a continually changing pH. For this reason an analysis of shaken cultures of h− bacteria was undertaken.

EXPERIMENTAL PROCEDURES

The majority of the methods used in this research are described in the first two papers of this series (Ryan and Schneider, 1948, 1949*a*). In addition, in order to create aerated conditions of growth, the 10-ml aliquots of medium were placed in 125-ml pyrex Erlenmeyer flasks to each of which had been sealed a calibrated pyrex test tube at an upward angle of about 30° at the side near the bottom. It was possible to shake and vigorously aerate the culture in the bottom of the flask and then, without removal from the system, to spill it into the side arm. In this position the tube was inserted into the adapter of a Klett-Summerson colorimeter for the measurement of optical density. We are indebted to Dr. R. Ballentine for suggesting this method. Before sterilization cotton plugs were inserted that had been covered with cheesecloth to prevent fibers from falling into the medium. After inoculation the flasks were shaken in a water bath at 37 C through an amplitude of 3 cm at an optimal rate of about 100 cycles per minute.

A variety of changes in the composition of the medium were made in order to develop optimal conditions for shaken growth. Since asparagine was found to shorten the lag period only slightly, it was omitted from the medium in all experiments reported in this paper. It was also found that sterile-filtered glucose or

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glucose autoclaved with the medium resulted in the same lag period, the same logarithmic rate of growth, and the same final crop of h+ or h− bacteria. This was also true when the concentration of the inorganic constituents of the medium was as described in the first paper of this series (Ryan and Schneider, 1948) or was halved. Similarly, shaking at from 80 to 120 cycles and through thrusts of from 1 to 3 cm did not influence growth differentially. Consequently, full-strength medium, autoclaved with glucose, was chosen as the standard for shaking under the conditions described.

The concentration of glucose, when varied from 0.025 to 0.5 per cent, did not influence the lag period or the logarithmic rate of growth of either h− or h+ bacteria. However, the total amount of glucose in the 10 ml of medium did have

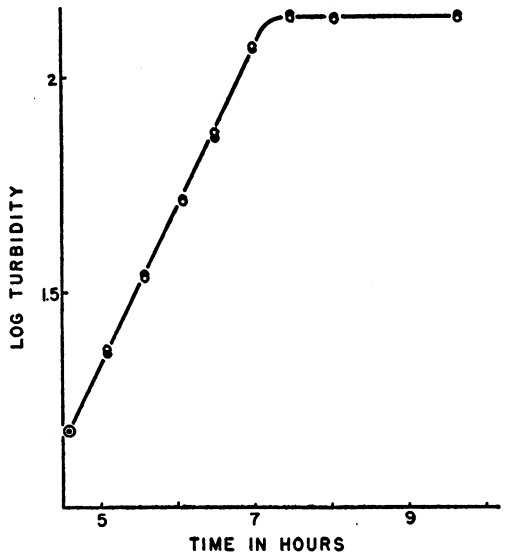

Figure 1. The growth curves of h+ and h− bacteria shaken in the absence of histidine and in the presence of 25 μg per ml, respectively. The open circles refer to h+ bacteria and the solid circles to h−.

a strong influence on the size of the final crop of bacteria. Moreover, it was found that at the completion of growth on 0.5 per cent glucose the pH of the medium dropped from 6.8 to about 4, despite aeration. On 0.05 per cent glucose the pH drops during growth from 6.8 to only about 6.5. Despite this small drop more than 5×10^8 bacteria are produced per ml. Apparently it is not aeration but sugar concentration that is critical in determining the amount of acid produced. Monod's (1942) medium, which also contains 0.05 per cent glucose, showed the same reduction in pH. It is, however, less well buffered and supported less bacterial growth. Consequently, we chose to use our medium with 0.05 per cent glucose.

RESULTS

Adaptation. Figure 1 shows the rate of growth of h+ bacteria in the absence of histidine and of h− bacteria in the presence of 25 μg per ml. The lag periods, loga-

rithmic rates of growth, and final populations of bacteria are the same. The final population size is limited by the amount of sugar present. When more sterile glucose is added in the stationary phase, growth is reinitiated. When, however, h− bacteria are grown on suboptimal concentrations of histidine, it is this substance that is limiting. When more sterile histidine is added to the culture, growth is reinitiated (figure 2).

The adaptation of h− cultures was studied on different concentrations of histidine under optimal aerated conditions. Figure 3 shows the time course of such

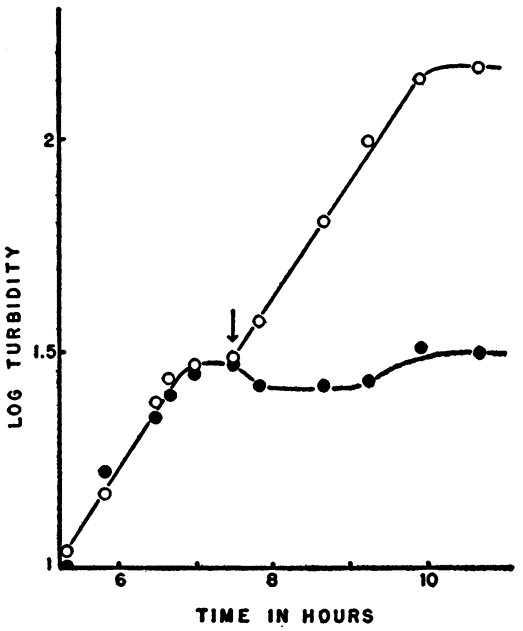

Figure 2. The effect of added histidine upon a shaken h− culture which had completed growth on a suboptimal concentration of histidine. Two cultures were allowed to grow in the presence of 0.8 μg histidine per ml until the stationary phase was achieved. At the time indicated by the arrow 0.1 ml of water, containing enough histidine to give a concentration of 25 μg per ml, was added aseptically to one culture. The control culture received just 0.1 ml of sterile water. The final growth achieved by the culture to which histidine was added is equal to the growth that the sugar in the medium would support.

adaptation. The rate of logarithmic growth is not dependent upon the initial concentration of histidine, but as this substance is depleted from the medium and becomes limiting, growth slows down and eventually stops. Those cultures that have received some histidine reach the stationary phase of growth within 9 hours. Then, after about 20 hours, the cultures on the lower concentrations of histidine and the one devoid of histidine begin adaptive growth, which is complete about 30 hours after inoculation. The time of adaptation is proportional to histidine concentration, as one would expect if the number of back mutations from h− to h+ were proportional to the number of h− bacteria present. The 30 hours required for the adaptation of the culture devoid of histidine is exactly the time required for the complete growth (5×10^9) of the 2 to 3 h+ bacteria

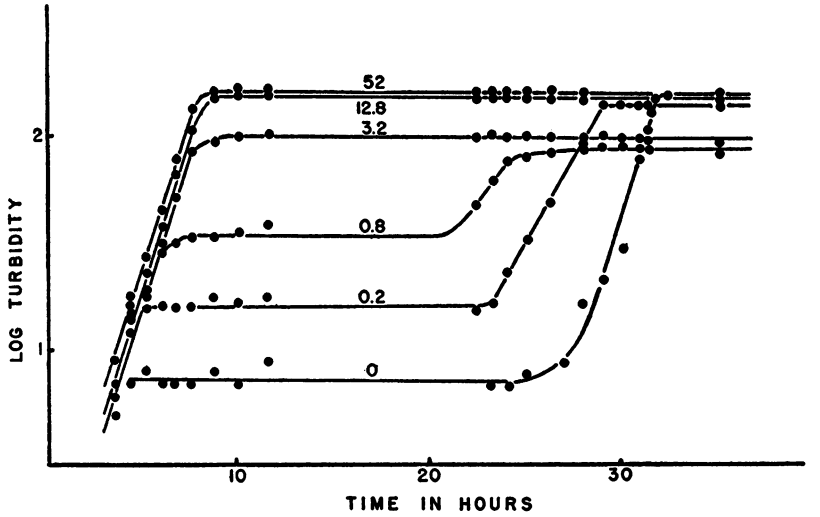

Figure 3. The time course of adaptation of shaken h− cultures on different concentrations of histidine. The numbers refer to the concentrations of histidine in μg per ml.

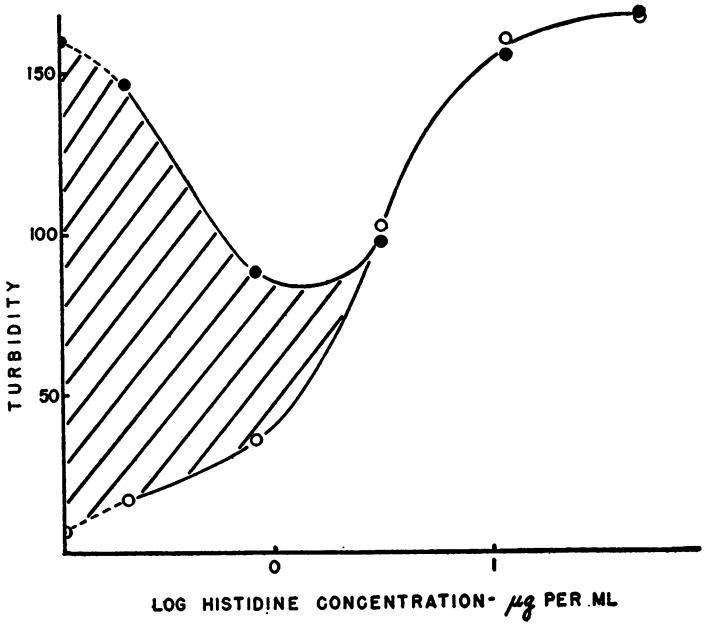

Figure 4. The relation of the amount of growth achieved with shaking before and after adaptation to histidine concentration. The lower curve is for growth at 10 hours and the upper curve for growth at 35 hours. The shaded area indicates the amount of adaptation. These data are the same as those shown in figure 3. The dotted lines go to the levels of growth on minimal medium devoid of histidine.

in the h− inoculum (2×10^7) if the generation time is the observed length of 55 minutes.

The extent to which adaptation takes place is, as it was in unshaken cultures, inversely proportional to the histidine concentration. Figure 4 shows this clearly.

Thus in aerated cultures, as in unaerated cultures, there is a dip in the adaptation curve, and the h+ bacteria, which are always present, do not grow to the extent expected. This depression of growth occurs despite the absence of the development of growth-limiting pH's in the course of growth. None of the aerated cultures had pH's of less than 6.5 after adaptation. Further, growth was not reinitiated by the sterile neutralization of any of these whole aerated cultures. Consequently, by shaking the cultures it is possible to study the inhibition of h+ by h− bacteria in the absence of the complication of the gradual development of a limiting pH during growth.

Test of inhibition. Filtrates suspected of containing inhibitors can be tested as they were in the studies reported in the previous paper (Ryan and Schneider, 1949*a*), by adding them to aerated cultures of h− and h+ bacteria in the logarithmic phase of growth. Sterile filtrates were prepared of unadapted and adapted

TABLE 1

The effect of filtrates of shaken h− cultures on the logarithmic rate of growth of h+ bacteria expressed as percentage of the rate prior to the addition

	H− CULTURE GROWN ON	
μg Histidine per ml	For 18 hr unadapted	For 30 hr adapted
0	107	93
0.2	102	107
0.4	97	112
0.8	100	96
1.6	100	100
3.2	87	100
6.4	100	104
12.8	100	94
51.2	91	91

h− cultures grown under aerated conditions in the presence of 0.05 per cent glucose and different concentrations of histidine. These filtrates were neutralized, concentrated under vacuum, and added, in volumes not over 10 per cent of the total culture, to h+ organisms. These were in the logarithmic phase of growth in the absence of histidine under aerated conditions. Table 1 shows the effect of filtrates from h− cultures on the growth of h+ bacteria. Unlike filtrates from unshaken cultures no inhibitory action is evident. The difference is not entirely, if at all, due to the aeration, for aerated cultures containing 0.5 per cent glucose were shown by this technique to produce an inhibitor (or inhibitors). However, aerated cultures grown on 0.05 per cent glucose do not produce stable inhibitors of the growth rate of h+ bacteria.

In order to test the possibility that an inhibitor from cultures grown on 0.05 per cent glucose affects the final level of growth without influencing the rate of growth, filtrates of h− cultures grown on different concentrations of histidine were inoculated with h+ bacteria with and without further supplementation with glucose. Table 2 describes the results of such an experiment. There was essentially

no growth on the unsupplemented filtrates, but it occurred when sugar was added. However, this growth was independent of the amount of growth that had occurred in the culture from which the filtrate had been prepared. Hence once again there was no evidence for the presence of an inhibitor in the h− filtrates. It is further evident from this table that the new growth was to the extent expected on the basis of the sugar added. In other words, there was no sugar left in the

TABLE 2

The final turbidity of h+ bacteria grown for 14 hours in neutralized filtrates prepared from shaken h− cultures adapted on different concentrations of histidine

μg HISTIDINE PER ML ORIGINAL H− CULTURE	UNSUPPLEMENTED FILTRATE	FILTRATE SUPPLEMENTED WITH 5 MG STERILE GLUCOSE (0.05 PER CENT)
0	7	140
0.8	9	137
1.6	8	129
3.2	11	139
6.5	4	125
25.8	0	123

TABLE 3

The growth of adapted cultures of h− bacteria resuspended in fresh shaken medium containing 25 μg histidine per ml

μg HISTIDINE PER ML IN ORIGINAL CULTURE	OPTICAL TURBIDITY OF ORIGINAL ADAPTED CULTURE	H+ PER 10^6 H− IN ORIGINAL ADAPTED CULTURE	OPTICAL TURBIDITY AFTER RESUSPENSION	H+ PER 10^6 H− AFTER RESUSPENSION
0	132	$>10^7$	125	$>10^7$
0.1	125	$>10^7$	125	$>10^7$
0.2	123	$>10^7$	125	$>10^7$
0.4	97	$>10^7$	128	$>10^7$
0.8	64	4.2×10^6	126	4.7×10^6
1.6	70	1.1×10^6	126	1.4×10^6
3.2	107	25.0	125	37.0
6.4	132	0.57	123	0.49
12.8	136	0.56	120	0.61
25.6	140	0.62	122	0.50

filtrates of adapted h− cultures, and this despite the fact that they had supported different amounts of growth.

It is still possible that the h− bacteria produce an inhibitor (or inhibitors) that cannot be found in the filtrate because it is absorbed by the h+ organisms. In order to test this notion 31-hour-old adapted h− cultures, grown on different concentrations of histidine, were washed and resuspended in medium containing 25 μg histidine per ml. Table 3 shows the levels of growth achieved in the new medium and the h− and h+ composition of the culture at the time of resuspension and after the new growth. There is no indication that the h+ bacteria were inhibited in their growth.

Limitation of growth. In order to examine the inference that the exhaustion of glucose from the medium, rather than an inhibitor (or inhibitors), limits the extent of adaptive growth, direct chemical determinations were made of the glucose remaining in the filtrates of adapted h−cultures. However, considerable difficulty was encountered with these methods. The copper reduction method of Somogyi (1945) was found to be interfered with by NH_4^+ in the concentrations used in our minimal medium. The ferricyanide method of Folin and Malmros (Umbreit *et al.*, 1945) and the iodine method of Wilstätter and Schudel (Brown and Zerban, 1941), although they allowed accurate determination of the glucose in our minimal medium, were interfered with by something in the culture filtrates from which it was impossible to recover added glucose accurately. Consequently, the nonspecific yeast assay used by Spiegelman (1947) was adopted and found to work satisfactorily. Manometric measurement of the CO_2 production during anaerobic glycolysis of h− culture filtrates in the presence of azide by starved *Saccharomyces cerevisiae* allowed recoveries of added glucose which averaged 95 per cent complete. By this method, which can detect as little as 0.05 mg glucose per ml, no sugar was found present in filtrates of h− cultures adapted on different concentrations of histidine. This finding confirmed the bioassay of the filtrates by h+ bacteria. Further, by both biological methods, filtrates of unadapted h− cultures were shown to contain glucose in inverse proportion to the amount of growth that had taken place. Where growth was complete on high concentrations of histidine, no glucose could be detected, although on lower histidine concentrations in which less and less growth occurred more and more glucose remained. It is this remaining glucose that is available for the adaptive growth of the h+ back mutants.

If these determinations are correct and adaptive growth in aerated cultures is limited by the exhaustion of glucose from the medium, then the addition of glucose to adapted cultures should reinitiate growth. Figure 5, curve 1, shows that sugar does reinitiate growth and that it was limiting at most histidine concentrations. However, at the shoulder of the adaptation curve, in the region of 3.2 μg histidine per ml, new growth does not occur and something else appears limiting. The addition of a second allotment of fresh sugar (curve 2) shows that this is not true, for growth is then reinitiated. But the second sugar addition does not initiate new growth in the region of 6.4 μg histidine per ml.

This unusual state of affairs has a simple interpretation. After adaptation cultures on all histidine concentrations are limited by the absence of glucose. When glucose is added, however, it is only the h+ bacteria that will grow—unless there is some histidine left over. The cultures grown on limiting histidine concentrations have no histidine left over and after adaptation consist for the most part of h+ bacteria. When fresh glucose is added, these bacteria grow. The cultures in histidine concentrations that yield optimal growth do not use up the histidine present but are limited by the exhaustion of glucose. These cultures consist almost entirely of h− bacteria; only a few h+ back mutants are present because of the absence of selection for them. When fresh glucose is added, the h− bacteria begin to grow again by using the histidine not consumed during their

previous growth. The h+ back mutants also reinitiate growth and remain in their original proportion. On a concentration of histidine like 3.2 μg per ml histidine has been used up and is limiting. Nevertheless, adaptation does not occur, and the h+ bacteria are present in very small numbers. When fresh glucose is added, they begin to grow. The h− bacteria, which cannot grow, consume glucose in maintenance, and by the time the small number of h+ bacteria have grown to visible turbidity, the sugar is exhausted. The second addition of glucose, however, finds a large number of h+ bacteria present which rapidly grow to completion before the still stationary h− organisms have wasted much glucose. On the other

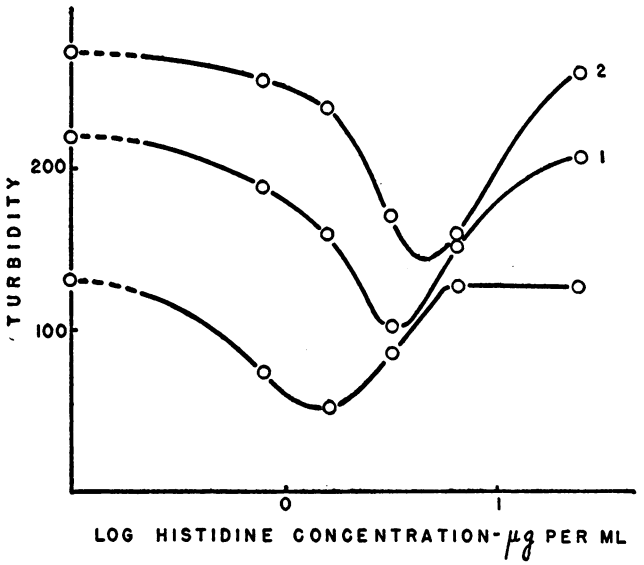

Figure 5. The effect of adding fresh glucose to shaken adapted cultures of h− bacteria grown on different concentrations of histidine. The lower curve shows the level of adaptive growth after 39 hours. At that time 5 mg sterile glucose (0.05 per cent) were added to each culture. Curve 1 shows the stationary level of new growth achieved 12 hours later. At that time another glucose addition was made and the new stationary level of growth achieved is shown by curve 2. The dotted lines go to the levels of growth on minimal medium devoid of histidine.

hand, the culture on 6.4 μg histidine per ml contains h− bacteria, and these grow, when the first sugar addition is made, until the excess histidine is exhausted. The small number of h+ back mutants present then begin to overgrow the culture, but before their proportion has increased appreciably the glucose is exhausted. The second glucose addition does not reinitiate growth of a culture started on 6.4 μg histidine for the same reason that the first addition did not reintiate growth of the culture grown on 3.2 μg histidine per ml.

It will be evident that this hypothesis predicts the data shown in table 4. In the first place, on either side of the dip in the curve the new growth involves a different type of bacterium. On low concentrations of histidine, h+ bacteria, which were present in large numbers, overgrow. On high concentrations, in which there was an excess of histidine, the h− and h+ bacteria both grow, retaining

their proportion. On 3.2 μg histidine per ml the h− bacteria do not increase upon the addition of glucose, for the histidine has been exhausted. Indeed, there is some death (but not lysis) of the h− bacteria. But the h+ bacteria do grow although just to the level of visible turbidity (ca. 5×10^6 per ml). Hence the turbidity of the culture does not increase until a second glucose addition is made. In this way there is a depression in the curve after the addition of glucose despite the fact that all shaken adapted cultures are limited in growth by the absence of glucose.

In constructing this interpretation it was assumed as demonstrated that adapted cultures have used up their glucose supply. They do this without achieving the same amounts of growth. It is thus still necessary to account for this apparent paradox. If h− bacteria in the stationary phase consume glucose without adding to the cell population, it is possible to understand the depression in

TABLE 4

The composition of shaken adapted h− cultures before and 7 hours after the addition to each of 5 mg sterile glucose (0.05 per cent)

ORIGINAL HISTIDINE CONCENTRATION, μg PER ML	NUMBER OF BACTERIA PER ML				H+ PER 10^6 H−	
	Adapted culture after 35 hr		After growth on added glucose		Adapted culture after 35 hr	After growth on added glucose
	h+	h−	h+	h−		
0	61×10^7	—	157×10^7	—	$>10^7$	$>10^7$
0.2	53×10^7	—	121×10^7	—	$>10^7$	$>10^7$
0.8	20×10^7	8×10^7	108×10^7	$<10^7$	2.5×10^6	$>10^7$
1.6	21×10^6	16×10^7	74×10^7	18×10^7	1.3×10^5	4.1×10^6
3.2	98×10^3	44×10^7	12×10^5	12×10^7	223	10^4
6.4	115	68×10^7	3×10^4	44×10^7	0.17	68
12.8	90	59×10^7	160	147×10^7	0.15	0.11
25.6	70	69×10^7	160	147×10^7	0.10	0.11

the adaptation curve and also all other facts that have been gathered in this connection. The following paper (Ryan and Schneider, 1949*b*) presents unequivocal evidence that this is the case.

SUMMARY

A method is described for the convenient measurement of the logarithmic rate of growth of *Escherichia coli* under conditions of shaking when the pH remains stable. Either the exhaustion of glucose or of histidine can be made to limit growth. Under these conditions adaptation of cultures of histidineless bacteria by the overgrowth of histidine-independent mutants occurs in the same way as in unshaken cultures. Similarly the adaptive growth of the histidine-independent bacteria is restricted by the presence of histidineless organisms on intermediate concentrations of histidine.

Unlike the situation in unshaken cultures, the production of inhibitory substances by the histidineless bacteria could not be demonstrated. Rather, the

growth of adapted cultures is limited by the exhaustion of glucose from the medium, and this despite the fact that unequal amounts of growth occurred on the different concentrations of histidine.

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