STUDIES ON THE NUTRITION OF DIM AND BRIGHT VARIANTS OF A SPECIES OF LUMINOUS BACTERIA

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A variant form of *Achromobacter fisheri* was frequently observed which differed from the original stock in a number of ways: namely, the colonies developed a conspicuous yellow pigment, the luminescence was more brilliant and lasting, and growth was more profuse than in the original stock. Self photographs of the two strains are shown in figure 1. It is evident that the variant was able to make more effective use of the nutrients provided in the medium than was the original stock. The experiments embodied in this paper were performed to determine the reason for the difference between the two strains.

Little is known concerning the chemical activities of luminous bacteria beyond the fact that they all grow well on complex media such as peptone or peptone-glycerol; that acid is produced in abundance from the various carbon sources, even under aerobic conditions (Hill, 1928); and that some will grow in inorganic media with a single simple organic source of carbon (Doudoroff, 1942).

The tendency for cultures of this species to become acid, coupled with the known sensitivity of their luminescence to acid, suggested that the difference between the two strains here studied might be (1) merely a difference in acid tolerance or (2) a difference in the rate of utilization of the acids formed as intermediaries in the decomposition of the complex nutrients. If the variant is better able to withstand acid than the original stock, utilization of the available nutrients might continue in the former case at a pH which would stop such usage in the latter. Thus, growth and luminescence might continue longer. If the variant is better able to utilize the acid intermediaries, it would also, by virtue of this fact, continue its activity for a longer time. The following analysis shows that both factors apparently play a part.

MATERIALS AND METHODS

Methods for the culture and preparation of suspensions of the bacteria and for the determination of their respiration and luminescence have been described elsewhere (Giese, 1941). The density of suspensions was measured with a densimeter of the type developed by Longsworth (1936). The pH was in all cases determined with a Beckman glass electrode. All cultures were grown and experiments conducted at approximately 25°C.

The agar plates used for growth of the bacteria contained 1 per cent glycerol, 0.6 per cent peptone, 0.2 per cent yeast extract, 0.2 per cent beef extract, 1 per cent $CaCO_3$, 2 per cent agar and 3 per cent NaCl. Liquid cultures were grown in 0.1 per cent peptone, 1 per cent glycerol and 3 per cent NaCl when no buffering was desired. The same nutrients with 1.5 per cent NaCl and M/8 solution of

the appropriate buffer were used when studies were to be conducted with liquid cultures at a given pH. Phosphate buffers were used in all cases.

In tests on the utilization of the organic acids for respiration, M/4 solutions of the acids neutralized with NaOH or $NaHCO_3$ were used in the side arms of the Warburg vessels giving, on dilution, approximately M/40 acid in the culture. Fresh solutions of all organic materials were made up for each series of experiments to minimize contamination.

Since for testing the utilization of various nutrients it is desirable to have bacteria containing relatively little stored material, cultures one day old were in each case washed from the plate in sea water and were centrifuged and suspended in fresh sea water and kept overnight in a refrigerator at about 5°C. This procedure was found by preliminary tests to exhaust the stored nutrients as effectively as aeration. On the following morning the cultures were again centrifuged and washed and suspended in buffered NaCl. For luminescence studies and in a few other cases mentioned in the text, 18–24 hour cultures were used.

EXPERIMENTAL

To determine the relation between pH and growth, bacteria of both strains were inoculated into liquid cultures buffered over the pH range 5.0 to 8.0 with peptone and glycerol as nutrients. It was found that the variant (hereafter designated Y) grew from 5.5 to 8.0 while the original stock (hereafter designated W) grew from 6.5 to 8.0. Growth in all cases was slight in the more acid cultures but no quantitative determinations were made. If Y grows better under more acid conditions than W because of its ability to utilize nutrients under acid conditions, its respiration should be greater than that of W under these conditions.

The relation between pH and respiration, occurring in the absence of nutrients in the medium and therefore presumably on the nutrients stored in the cells, was studied first. The respiration was found to increase with rise in pH as shown in figure 2. There is no essential difference between the respiration of the two strains over the pH range tested.

The respiration in the presence of nutrients in the suspension medium was therefore tried. It was found that neither Y nor W was capable of making effective use of peptone as the only organic nutrient at pH 6.0, for the respiration was little greater in presence of peptone than in its absence. An increase in usage occurred as the pH rose. At pH 7.5 the utilization of peptone was considerable and approximately equivalent for both strains. The data are summarized in figure 3. The difference in acid tolerance must reside in some property other than a difference in peptone utilization under acid conditions. However when the two strains are compared on the basis of the proportional increase in respiration over the endogenous rate on addition of peptone, Y has the advantage, the increase being roughly four to five-fold, whereas for W it is only a little over threefold for most of the pH range. Unfortunately different experiments are not completely consistent, indicating that the state of the bacteria may influence this response.

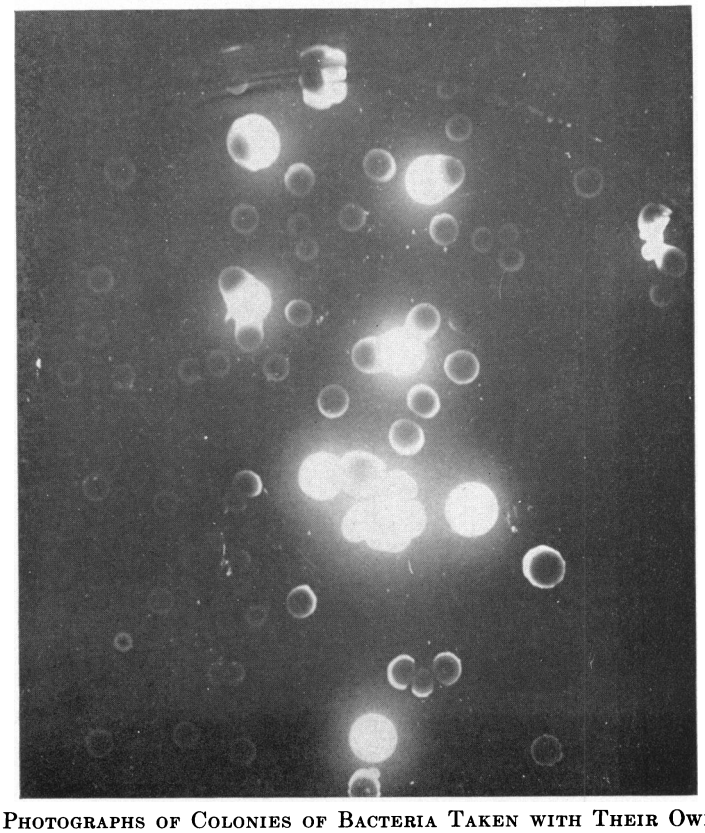

FIG. 1. PHOTOGRAPHS OF COLONIES OF BACTERIA TAKEN WITH THEIR OWN LIGHT
The bright colonies are the yellow variants, shown to be glowing brightly and still growing. Note that colonies of W are luminous, at least around the edges when they lie near Y; the side closest to Y in such cases is brightest.

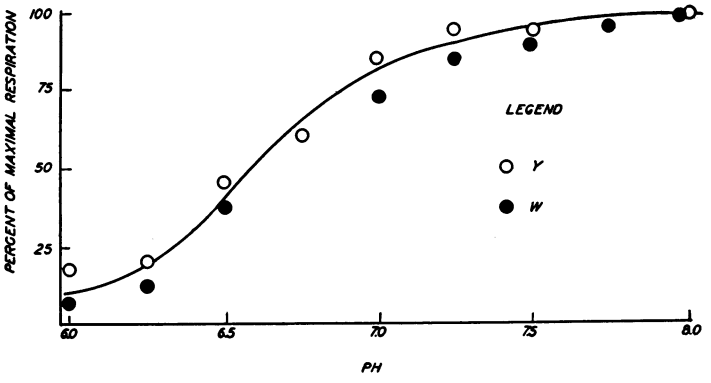

FIG. 2. THE RELATION BETWEEN pH AND ENDOGENOUS METABOLISM
Each of the points is the average of three separate experiments

In the following series of experiments it was found that there was a considerable difference in the utilization of glycerol by the two strains. Both Y and

W were able to use glycerol better, the higher the pH; for Y, considerable usage occurs over the range 6.5 to 8.0, for W, over the range 7.25 to 8.0. The experiments are summarized in figure 4. These experiments demonstrate that Y is better able to obtain energy for its respiration at a low pH than is W, which accounts in part for the ability of Y to grow under relatively acid conditions.

When either Y or W is supplied with glycerol as the only substrate, the unbuffered medium rapidly becomes acid, in both cases falling to about pH 6.0 in one-half hour, 5.5 in one hour and 5.0 in 7 hours. If the only difference between the two strains is the ability of Y to continue to use glycerol longer than W because of its lesser sensitivity to acid, one might expect that Y would merely continue to grow longer than W but that no other change in the medium would occur. Tests however disclosed that whereas agar plate cultures of W became and remained acid, those on which Y was growing became alkaline. Thus, even

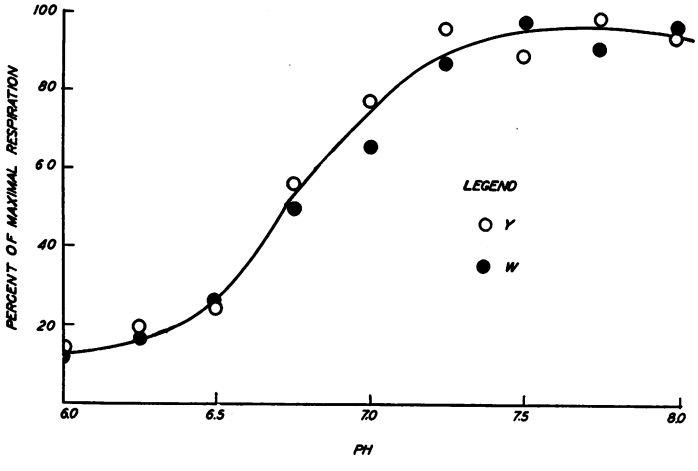

FIG. 3. THE RELATION BETWEEN pH AND PEPTONE UTILIZATION AS DETERMINED BY THE RATE OF RESPIRATION AT DIFFERENT pH VALUES
Averages of 4 experiments

on a plate on which W was streaked on one side, Y on the other, the pH was found to be about 6.0 on the extreme W side, about 7.5 to 8.0 on the extreme Y side. Also, in liquid cultures containing peptone and glycerol the pH of W cultures fell to 5.0 in four days, at which time growth and luminescence ceased, whereas cultures of Y became only slightly acid just after inoculation and later became somewhat alkaline. The results are given in figure 5. In these cultures the titratable acidity in cultures of W was twice that of Y on the third day after inoculation. It is therefore apparent that mere ability to use glycerol at a low pH is not the only difference between Y and W and that Y is either using the acids produced from glycerol or is neutralizing these acids with metabolites resulting from peptone metabolism.

To determine if the latter is true, the two strains were grown in liquid culture on peptone as the sole organic source. No difference in pH or in titratable

acidity was found between the two (fig. 5). Both Y and W produce alkali which, according to Hill (1928), is probably ammonia. The similarity in utilization of peptone in respiration by these two strains has already been pointed out (fig. 3).

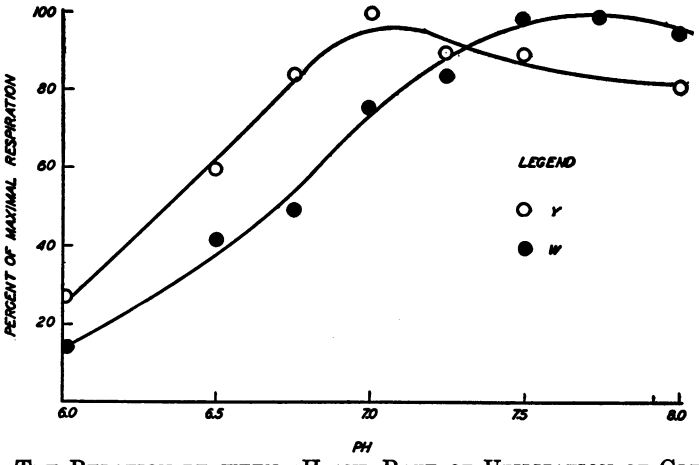

FIG. 4. THE RELATION BETWEEN pH AND RATE OF UTILIZATION OF GLYCEROL
Averages of three experiments

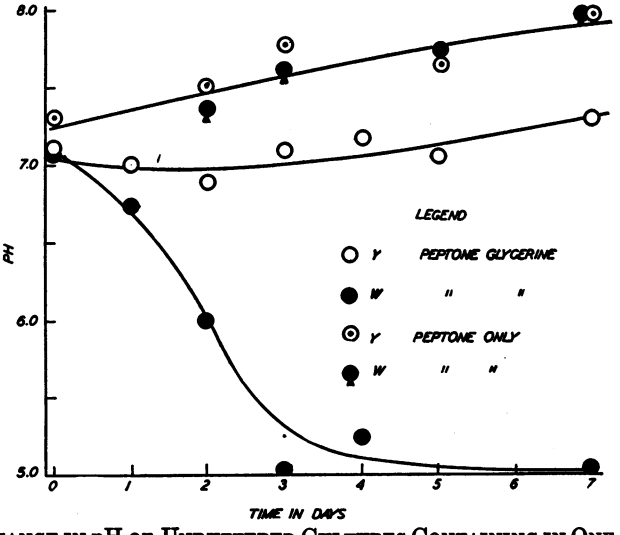

FIG. 5. THE CHANGE IN pH OF UNBUFFERED CULTURES CONTAINING IN ONE CASE PEPTONE ALONE, IN THE OTHER PEPTONE AND GLYCEROL
Average values

In the presence of glycerol it is possible that a difference in utilization of peptone by the two strains might occur.

The various experiments discussed above suggest that Y is better able than W to use acids produced from glycerol. It would therefore be interesting to determine which acids are used and to what extent. Unfortunately the inter-

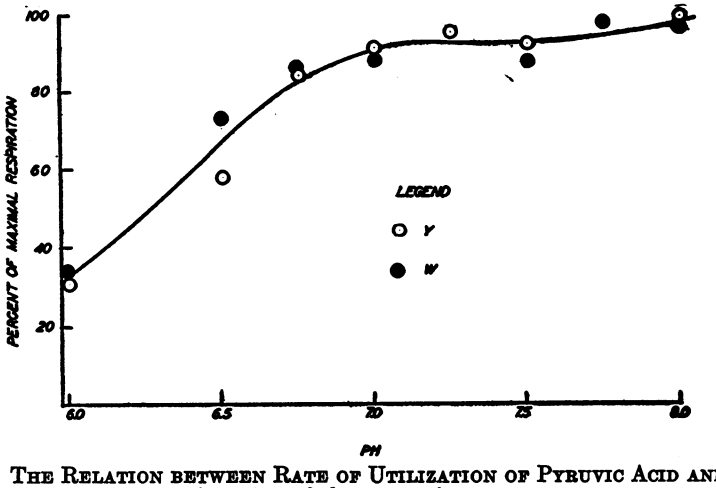

FIG. 6. THE RELATION BETWEEN RATE OF UTILIZATION OF PYRUVIC ACID AND pH
Averages of three experiments

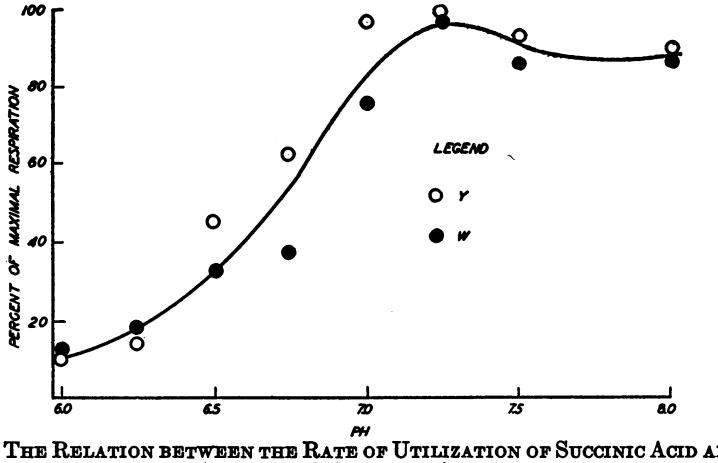

FIG. 7. THE RELATION BETWEEN THE RATE OF UTILIZATION OF SUCCINIC ACID AND pH
Averages of three experiments

TABLE 1

Relative rate of utilization of organic acids and peptone by Y and W strains of Achromobacter at pH 7.0

ACID	Y	W
Malic	2.4	1.9
Maleic	1.3	1.1
Fumaric	3.5	1.9
Succinic	3.9	1.9
Pyruvic	4.5	3.0

The figures were obtained by dividing the respiration in M/40 organic acid by the endogenous rate for an equivalent time. Glucose gives a value 15 times the endogenous rate. The usage varies with the state of the bacteria. Bacteria kept too long without nutrient do not respond.

mediary metabolism of these bacteria has not been adequately studied; however, on the basis of a survey of utilization of organic acids by microorganisms (Stephenson, 1939, pp. 190–197) a number of acids were chosen and tested. It was found that acetic, oxalic, lactic, tartaric, and citric were little if at all used, whereas maleic, malic, fumaric, succinic, and pyruvic were metabolized. In the utilization of the latter acids there were observed two differences between Y and W: (1) Y is able to use salts of some acids in a more acid medium than W; (2) Y is able to use some of them to a greater degree than W at a given pH. The data are summarized in table 1 and figures 6 and 7. In his recent study Doudoroff (1942 b) has shown that certain luminous bacteria produce fumaric, acetic, lactic and succinic acids, among other products in their anaerobic sugar dissimilation. If the species used here produces the same intermediate products it is surprising that Y in the presence of air is unable to use lactic and acetic acids.

DISCUSSION

The experimental data presented enable one to explain some of the differences between the dim and bright strains of *Achromobacter fisheri*. Both produce acid from glycerol, but since Y is better able to utilize glycerol even under acid conditions, it continues to grow after W has stopped. Secondly, since Y is able to use certain organic acids more effectively than W under acid conditions, it grows even after the supply of glycerol may have been depleted. Colonies of Y grow larger than colonies of W when the two are grown on the same plate as shown in figure 1. The more intense and continued luminescence of Y may also be in part due to the more efficient utilization of available nutrients by this strain.

The appearance of the bright variant Y is another example of the general tendency of the bacteria to adapt themselves to varied conditions (for references see Stephenson, 1939, ch. 11). The Y variant appeared frequently in liquid cultures of the original strain. In such cultures, in the absence of a buffer, the medium becomes acid rapidly and the appearance of a strain better able to tolerate the acid conditions is an adaptation. When the original strain was grown on agar plates and transfers were made every few days such changes were not observed. It should be pointed out that the degree of adaptability of the bacteria is limited. In unbuffered liquid cultures supplied with peptone and glucose the pH fell to about 4.9 or even lower in three to four days. Almost invariably the bacteria were killed, for growth seldom occurred when buffered nutrient medium was inoculated from such cultures. The bacteria were unable to adapt, either to the low pH or some other unfavorable conditions in the medium.

The mechanism by which the variation in *Achromobacter* occurs has not been investigated but since the Y strain appeared only several days after inoculation it seems probable that the change was a selection of naturally occurring variants such as those described in *Escherichia coli* by Massini (1907) rather than by a change in enzymes already present as described by Dubos (1940). Y was found to occur frequently in liquid cultures. The reversal to W was also found frequently on old plates and in old liquid cultures. Thus, on a plate 22 days old

containing only a small number of colonies, one large colony of Y was found to have five partial sectors of W.

A variation of the type Y to W in the production of dim mutants has been noted several times before, consequently it has been considered occasionally necessary in experimental work with luminescence to reisolate a brilliant strain from old cultures to be used as stock. Doudoroff (1938) investigated the nature of the dim mutants obtained in his cultures. He found that if these were grown in the presence of riboflavin and suspended in riboflavin solutions they equaled the brilliant strains in luminescence. This seemed an attractive explanation for the phenomenon described in this paper since the brilliant strain produces yellow pigment abundantly and the pigment diffuses into the medium, is soluble in water and fluoresces in the ultraviolet, suggesting riboflavin.[1] On this basis the W strain lacking the pigment might be considered dim because it is unable to produce the required riboflavin. However, when W was grown in the presence of extra riboflavin and suspended in solutions enriched with it, there was no essential change in luminescence. Secondly, the difference in pigmentation between the strains was found to be incidental for if both strains were grown on plates strongly buffered with phosphate salts at pH 8.0, both developed a pigment similar in color, neither developed pigment in acid cultures at first, Y developed such pigment when the cultures became alkaline.

The difference in luminescence of W and Y is apparently not an incidental one, due merely to pH and better availability of nutrients, for even when both strains are suspended at the same pH and adequate nutrient is supplied the luminescence of Y is 4 to 5 times greater than that of W. There is therefore some difference in the enzymatic system which controls luminescence. While it is possible that this difference is independent of the variation in sensitivity of the respiration to pH in the two strains, it is also possible that a fundamental alteration in some part of the enzymatic system underlies both.

It is interesting to point out one final difference between the two strains which might indicate some such fundamental change. It was found that when cultures of Y were suspended in solutions over a range of pH, precipitation of the bacteria occurred on the acid side of 6.8, whereas on the alkaline side very little or none occurred. Suspensions of W remained stable at all values of pH tried, except at or below 1.8 where denaturation may have occurred; suspensions also became clear at pH 10 due to cytolysis. It is clear that the surface properties of the two strains are different; Y acts as if it were charged negatively on the alkaline side of 6.8, W acts as if it were not so charged. For Y a covering with some protein is suggested, for W some other covering perhaps over the same underlying protein. Whatever the exact nature of this difference it indicates a characteristic modification of the cell properties with the variation from W to Y.

SUMMARY

1. A brilliant variant was observed to appear in liquid culture of *Achromobacter fisheri* when the medium was allowed to become acid. The luminescence of this form was 4 to 5 times that of the original strain under favorable conditions.

[1] W. J. van Wagtendonk, unpublished data.

2. On ordinary agar plates with calcium carbonate buffer, the variant developed a bright yellow-brown pigment, whereas the original strain did not. On buffered plates both strains developed the pigment at pH 8.0, neither on acidified plates.

3. Peptone increases the respiration of both strains and the sensitivity of each to pH is similar in both cases.

4. The variant uses glycerol more effectively than the original strain under acid conditions.

5. Neither strain uses a number of organic acids, both use succinic, fumaric, malic and pyruvic acids readily. The increase in respiration obtained by adding the acid is greater for the variant strain than for the original.

6. The variant strain behaves as if the individuals were charged, for precipitation occurs on the acid side of pH 6.8; the original strain is not precipitated at any viable pH.

7. Dim mutants regularly occur in cultures of the bright strain under certain conditions.

8. The bright strain may be considered a variant which adapts the species to the acid conditions unfavorable to the original strain. The time required tor the appearance of a population of variants suggests that not adaptive enzyme formation but selection of naturally occurring variants accounts for the change.

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