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UTILIZATION OF AROMATIC AMINO ACIDS BY *HYDROGENOMONAS FACILIS*

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ABSTRACT

DECICCO, B. T. (Rutgers, The State University, New Brunswick, N.J.), AND W. W. UMBREIT. Utilization of aromatic amino acids by *Hydrogenomonas facilis*. J. Bacteriol. 88:1590-1594. 1964. —An auxotrophic mutant of *Hydrogenomonas facilis* was isolated which requires tryptophan, phenylalanine, and *p*-aminobenzoic acid (PABA) for growth. With glucose as the main carbon and energy source, the quantitative requirements for tryptophan and PABA were at normal microgram levels, but the requirement for phenylalanine was very large and approached substrate concentrations. The large phenylalanine requirement is due to a rapid oxidation and degradation of phenylalanine by the mutant. The utilization of both phenylalanine and glucose is adaptive, and the presence of phenylalanine partially inhibits the induction of the glucose-utilizing system. Wild-type *H. facilis* can utilize either phenylalanine or tyrosine for growth. Tracer studies indicated that during growth on phenylalanine, the aromatic ring is opened and degraded. Wild-type cells grown on either phenylalanine or tyrosine can oxidize phenylalanine, tyrosine, or phenylpyruvate without a lag. Another inducible pathway enables *H. facilis* to utilize either quinate or 3,4-dihydroxybenzoate for growth, and sequential adaptation studies revealed that quinate is converted to 3,4-dihydroxybenzoate during its degradation. Mutants may be obtained which can also utilize 2,5-dihydroxybenzoate for growth.

Since the original isolation of *Hydrogenomonas facilis* and the subsequent studies performed on this organism by Schatz and Bovell (1952), the heterotrophic nature of its metabolism has been investigated primarily in relation to its autotrophic capacities. Thus, Atkinson (1955) demonstrated the adaptive nature of the oxidations of glucose and Kreb's cycle intermediates, using cells grown both autotrophically and heterotrophically. Kluyver and Manten (1942) and Wilson et al. (1953) demonstrated the simultaneous oxidation of hydrogen and organic substrates. More recently, Goodman and Rittenberg (1964) furnished evidence that some hydrogen bacteria can exhibit simultaneous autotrophic and heterotrophic growth when both types of substrates are available.

The studies to be described are mainly concerned with certain unusual aspects of the heterotrophic metabolism of *H. facilis* which were revealed while working with an auxotrophic mutant of this organism. The mutant was found to require phenylalanine, tryptophan, and *p*-aminobenzoic acid (PABA). Phenylpyruvate can be substituted for the phenylalanine requirement, and indole or anthranilate could replace tryptophan. Quantitative studies concerning the three requirements led to the findings described below.

MATERIALS AND METHODS

Organisms. A culture of *H. facilis* was originally obtained from R. H. Burris, Department of Biochemistry, University of Wisconsin, and has been subsequently maintained for 4 years. An auxotrophic strain of this organism requiring phenylalanine, tryptophan, and PABA was derived from the wild type by use of a modification of the penicillin technique developed by Lederberg and Zinder (1948) and Davis (1948).

Media and cultural conditions. For growth studies with the wild-type organism, a minimal salts medium containing between 0.05 and 0.2% of an organic substrate was employed. The minimal salts medium contained the following components: 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% $(NH_4)_2SO_4$.

For studies with the auxotrophic strain, 10 μg/ml of tryptophan, 1 mμg/ml of PABA, and the indicated concentrations of phenylalanine were added to the minimal growth medium.

When employed, Trypticase Soy Broth (BBL) was prepared by dissolving 15 g of the powder in 1 liter of distilled water. Unless otherwise noted,

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all cultures were incubated at room temperature with shaking.

Growth measurements. Growth was determined turbidimetrically by use of either a Bonet, Maury, and Jouan Biophometer, or by measuring 10-ml cultures in standardized 10-mm growth tubes with a Bausch & Lomb Spectronic-20 colorimeter at 540 mμ.

Measurement of O_2 uptake. To assay for the presence of adaptive enzymes, standard Warburg methods (Umbreit, Burris, and Stauffer, 1957) were employed. The tested substrates were added to a final concentration of 0.006 M. All Warburg studies were performed at 28 C with KOH in the center wall.

Isotope studies. To demonstrate aromatic ring breakage, uniformly labeled C^{14}-L-phenylalanine was added to a cell suspension in a Warburg vessel. The center well contained filter paper plus KOH. The flasks were shaken overnight at 28 C, and trichloroacetic acid was added to the cell suspension to a final concentration of 6%. After an additional 30-min shaking period, the filter paper was removed, and washed in 10 ml of alkaline water to obtain labeled CO_2 in solution. The cell-suspension precipitate was removed from the flask and centrifuged; the supernatant fluid was removed, and the sediment was suspended in water. Samples of the three fractions were then placed onto planchets, dried, and analyzed for radioactivity with a Tracerlab Sc-70 compumatic scaler.

Chemicals. L-Phenylalanine, sodium phenylpyruvate, quinic acid, and indole were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. L-Tyrosine and PABA were purchased from Merck & Co., Inc., Rahway, N.J.; anthranilic acid was obtained from Eastman Organic Chemicals, Rochester, N.Y. Uniformly labeled C^{14}-L-phenylalanine was a product of Volk Radiochemical Corp., Skokie, Ill.

RESULTS

After the requirements of the auxotrophic mutant of *H. facilis* were found to be phenylalanine, tryptophan, and PABA, it was found that, in broth containing 0.2% glucose, 20 μg/ml of phenylalanine and tryptophan, and 5 mμg/ml of PABA, the final growth yield was definitely suboptimal as compared to growth of the wild type on glucose alone. Quantitative growth studies revealed that with 0.2% glucose as the major carbon and energy source, the limiting concentration of tryptophan required for maximal growth was about 5 μg/ml, and for PABA was below 1 mμg/ml. The concentration of phenylalanine required for optimal growth, however, was in excess of 200 μg/ml (Fig. 1, glucose curve). This high quantitative requirement for phenylalanine suggested that when both phenylalanine and glucose are available to the mutant, the phenylalanine was utilized in preference to the glucose. After removing and degrading the phenylalanine present, the mutant would be incapable of growing on the available glucose due to its phenylalanine requirement for protein synthesis.

This hypothesis was explored by replacing glucose with other organic substrates to determine whether the phenyalanine requirement was quantitatively reduced (Fig. 1). When 0.2% glutamate was substituted for glucose, the requirement for phenylalanine was drastically reduced. Substituting 0.05% tryrosine for glucose also lowered the phenylalanine requirement. The bottom curve (control) represents growth of the mutant when only the three required substances were present. Thus, it represents growth of the mutant on the supplied phenylalanine, because other studies revealed that neither tryptophan nor PABA are broken down for carbon and energy by *H. facilis*.

Wild-type *H. facilis* grew by utilizing either phenylalanine or tyrosine as its sole carbon and energy source. The utilization of these amino acids was adaptive, because cells grown on glucose

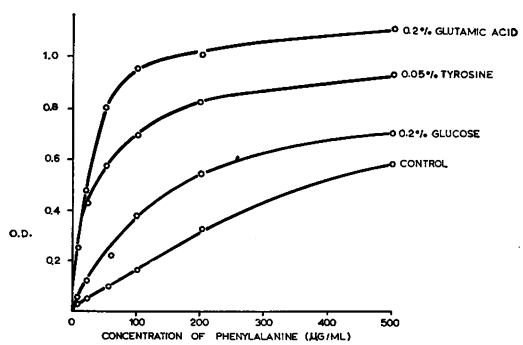

FIG. 1. *Maximal growth obtained by a multiple auxotroph of Hydrogenomonas facilis with various concentrations of phenylalanine. Each tube contained salts, 10 μg/ml of tryptophan, 1 mμg/ml of PABA, the indicated concentration of phenylalanine, and the tested substrate. The control tubes contained no additional substrate.*

or glutamate showed no capacity to oxidize either of the aromatic compounds. Growth on either phenylalanine or tyrosine allowed the organism to oxidize phenylalanine, tyrosine, or phenylpyruvate without a lag. Phenylalanine and phenylpyruvate were oxidized at approximately equal rates, and tyrosine was oxidized somewhat more slowly. Growth on phenylpyruvate did not occur, even though phenylpyruvate can be oxidized and substituted for the phenylalanine requirement of the mutant, indicating that it can be converted to phenylalanine.

The fastest growth rates and best cell yields with wild-type *H. facilis* were obtained in a medium containing protein hydrolysates. In these media containing amino acids, the addition of glucose did not affect the growth of the culture. This suggested a preferential utilization of amino acids over glucose. Both glucose and phenylalanine were oxidized adaptively; thus, it was possible to observe the relative rates of their oxidation under various conditions. When cells were grown in a medium containing both glucose and phenylalanine (Table 1), as in Trypticase Soy Broth, the phenylalanine-utilizing system was fully induced, but the glucose-utilizing system was only at approximately 30% of its maximal activity. Assuming the Trypticase component contained about 5% phenylalanine and 3.5% tyrosine, there was still about twice as much glucose present (by weight) in Trypticase Soy Broth as these two amino acids combined. Other studies have indicated that even when the concentration of phenylalanine was reduced to 0.02%, and the concentration of glucose was 0.3%, the phenylalanine-utilizing system was still fully induced, and the glucose system was not.

The results of an experiment to determine whether the aromatic ring of phenylalanine is opened and utilized during its degradation by *H. facilis* are shown in Table 2. Though three of the nine carbon atoms of phenylalanine were in the side chain, if more than one-third of the added C^{14} was recovered as CO_2, the ring must have been degraded. The results indicated that over 50% of the supplied carbon was liberated as CO_2, and hardly any was left in the supernatant fluid, where a cyclic waste product would be likely to appear; it seemed evident that ring breakage occurred. The experiment was repeated three times and the percentage of counts recovered varied between 95 and 104% in each case. The CO_2 fraction never accounted for less than 50% of the total recovered counts.

H. facilis was also found to utilize either quinic acid or 3,4-di-OH benzoic acid as sole carbon and energy sources. Manometric studies revealed that the utilization of these substrates was also adaptive. When grown on quinate, cells oxidized both quinate and 3,4-dihydroxybenzoate (DHB), at approximately equal rates but, when grown on 3,4-DHB, quinate was not oxidized (Table 3). The breakdown of quinate thus appeared to involve 3,4-DHB as an intermediate. Mutants of *H. facilis* may be selected which are also capable of utilizing 2,5-DHB for growth.

TABLE 1. *Oxidation of glucose and phenylalanine by Hydrogenomonas facilis*

Growth medium*	Substrate tested	Net O_2 uptake
		μliter/hr
Glucose-salts	Glucose	80
	Phenylalanine	0
Phenylalanine-salts	Glucose	0
	Phenylalanine	228
Trypticase Soy Broth	Glucose	26
	Phenylalanine	222

* Final optical density of cells, 1.5 (540 mμ).

TABLE 2. *Degradation of uniformly labeled C^{14}-phenylalanine by Hydrogenomonas facilis*

Fraction	Counts*	Per cent of total recovered counts
CO_2	22,300	57
Trichloroacetic acid precipitate + cells	15,650	40
Supernatant fluid	1,400	3

* Total recovered counts = 39,350. Per cent recovery = 95%.

TABLE 3. *Oxidation of quinate and 3,4-dihydroxybenzoate (DHB) by Hydrogenomonas facilis*

Growth medium*	Substrate tested	Net O_2 uptake
		μliter/hr
Quinate-salts	Quinate	348
	3,4-DHB	330
3,4-DHB-salts	Quinate	0
	3,4-DHB	340

* Final optical density of cells, 1.5 (540 mμ)

DISCUSSION

Although the quantitative requirement of the mutant for phenylalanine could be reduced by substituting glutamate for glucose, even with glutamate, which is readily and constitutively utilized by *H. facilis*, the requirement for phenylalanine is still high. It appears that the degradation of phenylalanine or tyrosine is highly desirable to *H. facilis*, because the metabolic enzymes which perform this task are rapid and are fully induced, even when phenylalanine and tyrosine are only minor components of a complex medium. Growth studies support this concept, because growth on phenylalanine is faster, and the cell yield is equal to or greater than that of any other single substrate tested.

The presence of phenylalanine did not completely suppress the utilization of glucose. If this were the case, the amount of growth of the mutant should be dependent only on the concentration of phenylalanine and independent of the presence of glucose. When glucose is present there is a greater cell yield than in its absence (Fig. 1), especially when the concentration of phenylalanine is low. The reason for the partial inhibition of glucose utilization by phenylalanine is not known.

There is a considerable amount of evidence that *H. facilis* is closely related to some members of the genus *Pseudomonas*. Tabak (1962) studied the utilization of some carbohydrates and Kreb's cycle intermediates, and concluded that on the basis of morphological, nutritional, and biochemical studies, *H. facilis* is closely related to *Pseudomonas putrefaciens*. Pootjes (1963) reported that deoxyribonucleic acid studies also placed *H. facilis* with the pseudomonads. The results reported in this paper agree with this taxonomic relationship, because at least some pseudomonads, as well as *H. facilis*, are capable of degrading and using phenylalanine (Chapman and Dagley, 1960) and quinate as sole carbon and energy sources. Also, in contrast to most bacterial species, which cannot convert phenylalanine to tyrosine, *H. facilis* appears to possess the enzyme phenylalanine hydroxylase which performs this conversion. Thus, the mutant does not require tyrosine, and cannot substitute tyrosine for phenylalanine. Phenylalanine hydroxylase is also common in the pseudomonads (Guroff and Ito, 1963). Moreover, the degradation of quinate by *Pseudomonas* has been shown to involve 3,4-DHB as an intermediate (Rogoff, 1958), and sequential adaptation studies with *H. facilis* suggest this same intermediate.

2,5-DHB did not support growth of wild-type *H. facilis* within a period of 48 hr. Eventually, growth was obtained; a colony from this variant culture was isolated, and its progeny was carried for several transfers on Trypticase Soy Agar. When subsequently tested for its ability to utilize 2,5-DHB, good growth was obtained within 24 hr. Thus, the variant appears to be genetically distinct from the parent strain.

In the facultative autotrophic hydrogen bacteria, the metabolic events occurring seem to depend largely on the species of carbohydrates and amino acids present. This may also extend to their autotrophic capacities, for the nature of any organic substrate present seems to affect the organism's metabolism at any specific period. Thus, some organic substrates, which appear to be utilized relatively slowly, allow simultaneous autotrophic and heterotrophic reactions (Wilson et al., 1953; Goodman and Rittenberg, 1964); more readily utilizable compounds, such as glutamate or phenylalanine, reduce the amount of H_2 which can be used for energy. In the presence of these rapidly utilized substrates, the organism would be living an essentially heterotrophic existence. Preliminary evidence obtained with the phenylalanine-requiring mutant suggests that the amount of growth obtained when both phenylalanine and H_2 are supplied is only slightly more than when phenylalanine alone is present (plus small amounts of tryptophan and PABA). Thus, H_2 appears to behave like glucose, because both are either second choices as substrates, or are taken into the cells more slowly than is phenylalanine. No way has been found to eliminate the utilization of the supplied phenylalanine as a carbon and energy source in the presence of H_2; therefore, the mutant must be said to be a strict heterotroph.

ACKNOWLEDGMENT

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LITERATURE CITED

ATKINSON, D. E. 1955. The biochemistry of Hydrogenomonas. II. The adaptive oxidation of organic substrates. J. Bacteriol. **69**:310-315.

CHAPMAN, P. J., AND S. DAGLEY. 1960. Bacterial

oxidation of phenylalanine and phenylacetic acid. Biochem. J. **75**:6P.

DAVIS, B. D. 1948. Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc. **70**:4267.

GOODMAN, N. S., AND S. C. RITTENBERG. 1964. Simultaneous autotrophy and heterotrophy in hydrogen bacteria. Bacteriol. Proc., p. 112.

GUROFF, G., AND T. ITO. 1963. Induced soluble phenylalanine hydroxylase from *Pseudomonas* sp. grown on phenylalanine or tyrosine. Biochim. Biophys. Acta. **77**:159–161.

KLUYVER, A. J., AND A. MANTEN. 1942. Some observations on the metabolism of bacteria oxidizing molecular hydrogen. Antonie von Leeuwenhoek J. Microbiol. Serol. **8**:71–85.

LEDERBERG, J., AND N. D. ZINDER. 1948. Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc. **70**:4267–4268.

POOTJES, C. 1963. Studies on a *Hydrogenomonas facilis* bacteriophage and *H. facilis* deoxyribonucleic acid. Ph.D. Thesis, Rutgers, The State University, New Brunswick, N.J.

ROGOFF, M. H. 1958. An aromatic intermediate in the bacterial oxidation of quinic acid. J. Gen. Microbiol. **19**:330–339.

SCHATZ, A., AND C. BOVELL. 1952. Growth and hydrogenase activity of a new bacterium, Hydrogenomonas facilis. J. Bacteriol. **63**:87–98.

TABAK, R. 1962. Studies on the facultative autotrophic bacteria. M.S. Thesis. Rutgers, The State University, New Brunswick, N.J.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques. Burgess Publishing Co., Minneapolis.

WILSON, E., H. A. STOUT, D. POWELSON, AND H. KOFFLER. 1953. Comparative biochemistry of the hydrogen bacteria. I. The simultaneous oxidation of hydrogen and lactate. J. Bacteriol. **65**:283–287.