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Interchangeability of Quinolinic and Nicotinic Acids as Growth Factors for a *Pseudomonad* Oxidizing Nicotinic Acid

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SUMMARY: A pseudomonad obtained from a nicotinic acid enrichment culture had a growth-factor requirement satisfied by high concentrations of nicotinic acid or by minute amounts of quinolinic acid, 3-hydroxyanthranilic acid, or nicotinamide. Nicotinic acid, kynurenin sulphate, and probably 3-hydroxyanthranilic acid, can serve as sole carbon sources. The oxidation of nicotinic acid is adaptive. The finding that low concentrations of nicotinic acid fail to satisfy the growth-factor requirement is discussed.

The paucity of records of auxotrophy in pseudomonads may reflect the selection exercised by isolation media devoid of growth factors. Pseudomonads requiring aneurin were isolated from enrichments with uracil and thymine. The utilization as carbon sources of metabolites of the anthranilic acid-tryptophan-nicotinic acid series may prove of taxonomic value in *Pseudomonas*.

Studies of the oxidation of tryptophan (Stanier & Tsuchida, 1949) and of other aromatic compounds (Stanier, 1950; Happold, 1950) by bacteria suggest that dissimilation of these compounds may proceed via pathways (in reverse) common to biosynthesis. Such applications of micro-organisms as tools for investigating intermediary metabolism prompted the present investigation of isolation methods. This paper includes a discussion of enrichment techniques and a report of the investigation of oxidative and biosynthetic pathways in a pseudomonad isolated by a modification of the usual enrichment culture technique.

The usual method of isolation is to enrich an inorganic medium with the substrate of interest, inoculate with soil, and eventually obtain the desired organism from the enrichment culture. This procedure is limited in that it selects against organisms which are auxotrophic (i.e. growth-factor-requiring). The importance of auxotrophy is underscored by the experience of den Dooren de Jong (1926) who failed to obtain hexanol-oxidizing bacteria by the simple enrichment technique. Foster (1944) later showed that many photosynthetic pseudomonads (cf. van Niel, 1944) utilize hexanol, and they require growth factors (Hutner, 1950). *Euglena gracilis*, an algal flagellate which also can

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utilize hexanol and grow heterotrophically in darkness, requires aneurin and vitamin B₁₂ (Hutner & Provasoli, 1951).

Compounds involved in the biosynthesis of nicotinic acid, i.e. anthranilic acid, tryptophan and nicotinic acid, served as test substrates in our studies. The biosynthesis of these compounds is under scrutiny in *Neurospora* and in animals, and material for comparison is thus available. Furthermore, as Koser & Baird (1944) had found that nicotinic acid was an excellent enrichment substrate for pseudomonads, it became of interest to determine whether the provision of growth factors in minimal amounts would permit the isolation of auxotrophic pseudomonads. The structures of the various compounds related to nicotinic acid which are referred to in this paper are shown in Fig. 1 (p. 280).

One of our enrichment cultures yielded a pseudomonad having a growth-factor requirement satisfied by high concentrations of nicotinic acid (pyridine-3-carboxylic acid) or by quinolinic acid (pyridine-2, 3-dicarboxylic acid) in minute concentrations. Since this bacterium has been applied as an assay organism for quinolinic acid (Jakoby & Bonner, 1951), and since quinolinic acid has been reported to be a possible intermediate in the biosynthesis of nicotinic acid, the physiology of the organism merits special description.

METHODS

Unless otherwise stated, the cultural methods were those previously described (Hutner, 1950).

Isolation procedure. The isolation steps were the following: (a) inoculation of the enrichment medium with soil or mud; (b) inoculation of a flask of enrichment medium with a loopful of culture from a; (c) streaking growth from b on to enrichment agar; (d) picking colonies on to agar slopes of enrichment medium; and (e) selection of cultures oxidizing the substrate in liquid media. Flagella staining by the silver method of Novel (1939) proved simple and reliable for identifying polar flagellate rods.

Culture media. The basal medium (Table 1) finally adopted both for enrichment cultures and for pure culture studies permitted good growth when supplemented with appropriate substrates. The substrate employed in growth-factor experiments was sodium succinate.6H₂O (0.2%) and NaH glutamate (0.125%). Ethylenediamine tetra-acetic acid (EDTA) was used as a metal carrier to insure the availability of essential but precipitable metals (Hutner, Provasoli, Schatz & Haskins, 1950). The media for enrichment cultures were supplemented with growth factors at the following concentrations (mg./100 ml. final volume): adenine, 1.0; uracil, 1.0; DL-methionine, 1.0; aneurin, 0.05; Ca pantothenate, 0.05; nicotinic acid, 0.05; pyridoxine, 0.02; riboflavin, 0.02; folic acid, 0.01; biotin, 0.002.

Basal media and stock solutions of nutrients were preserved with a mixture consisting of (v/v) 1 part chlorobenzene, 2 parts *n*-butyl chloride, and 1 part 1:2-dichloroethane; autoclaving removed the preservative. No contaminations in stock solutions were encountered while using it over a 2-year period.

Table 1. *Basal medium for growth-factor studies*

	g.
K_2HPO_4	0.02
Ethylenediamine tetra-acetic acid (EDTA)	0.05
$MgSO_4 \cdot 7H_2O$	0.08
NH_4Cl (omitted in enrichment media)	0.02
Metals (mg.): Ca, 2.0; Zn, 5.0; B, 2.0; Mn, 1.4; Fe, 1.0; Mo, 0.6; Cu, 0.4; Co, 0.4.	

Distilled water to 100 ml.; pH adjusted to 6.6–6.8 with KOH. The concentration of metals refers to the metal content of the salts used. Calcium was dispensed from a solution made by dissolving $CaCO_3$ in a minimal quantity of HCl. Free EDTA is sparingly soluble but readily dissolves as the medium is neutralized.

L-Kynurenin sulphate and 3-hydroxyanthranilic acid were sterilized by filtration. An Eastmen preparation of quinolinic acid was re-crystallized from 40 % acetic acid until its nicotinic acid content as measured with *Lactobacillus arabinosus* was insignificant.

Fig. 1. Structural formulae of compounds used.

Culture conditions. Enrichment media were distributed in shallow layers in 125 ml. flasks and incubated at 22–30°. Growth experiments with the 'quinolinic' pseudomonad were conducted in 50 ml. flasks containing 10 ml. of medium and incubated at 30°. As was anticipated from the oxidative character of the organism, growth was greater and more rapid in shaken cultures. Stock cultures were stored at 6° on agar slopes covered with paraffin oil. The 'quinolinic' pseudomonad is in the American Type Culture Collection (No. 108,38).

Manometric technique. O₂ and CO₂ were measured by the direct method of Warburg. Cells for these experiments were incubated on a shaker for 2 days at 30°. The cells were collected by centrifugation, washed twice in M/60 phosphate (pH 7.0), and suspended in the same buffer.

RESULTS

The 'quinolinic' pseudomonad

Growth-factor requirement. The 'quinolinic' pseudomonad was obtained from a culture enriched with 0.2% nicotinic acid. Its auxotrophy was detected when the succinate glutamate medium failed to support growth. Growth was obtained when this medium was supplemented with relatively large quantities of nicotinic acid. Quinolinic acid and 3-hydroxyanthranilic acid were efficacious in extremely small amounts (Fig. 2). L-Tryptophan, kynurenin sulphate and anthranilic acid had very slight activity; indole was inhibitory.

Fig. 2. Utilization of compounds of the anthranilic acid-tryptophan-nicotinic acid series as growth factors in succinate glutamate medium. The insert graph (lower right-hand corner) is an expansion of the quinolinic acid and nicotinamide curves in the large graph.

Carbon sources. When the organism was grown on the usual succinate glutamate medium with quinolinic acid as the 'vitamin source', the organisms were not adapted to the oxidation of nicotinic acid or other intermediates in its synthesis. Adaptation to oxidize nicotinic acid required approximately 2 hr. Bacteria grown on nicotinic acid as both carbon and vitamin source were adapted to the oxidation of nicotinic acid alone. Although 3-hydroxyanthranilic acid was not tested as a carbon source (because of its scarcity) the increase in oxygen uptake in its presence (Table 2) indicated that it probably can function as such.

Nicotinic acid at 10 mg./100 ml. allowed visible growth. L-Tryptophan,

kynurenin sulphate and anthranilic acid allowed slow growth at higher concentrations. Nicotinamide and quinolinic acid would not serve as carbon sources.

The theoretical R.Q. for complete oxidation of nicotinic acid is 1.1. The R.Q. actually observed for concentrations of nicotinic acid varying from 0.1 to 3 μM . was 0.65–0.66. In the presence of 2:4-dinitrophenol, a reagent considered to uncouple oxidation from assimilation, the R.Q. was increased to 0.87, with

Table 2. *Manometric measurements of the oxidation of substrates related to quinolinic acid*

Substrate	$\mu\text{mole O}_2/\mu\text{mole}$ carbon source
Quinolinic acid	0
Nicotinamide	0.2
3-Hydroxyanthranilic acid	2.6
Kynurenin. SO_4	3.6
Nicotinic acid	3.6

Final values were taken when the oxidation rate was equal to that of the endogenous respiration. Small amounts of nicotinic acid in the range tested (0.1 μM . to 3 μM .) were oxidized to the same extent as larger amounts. The bacteria for this experiment were grown with quinolinic acid as vitamin source and succinate + glutamate as substrate.

a concomitant increase in oxygen consumption from 3.6 $\mu\text{mole O}_2$ to 5.1 $\mu\text{mole O}_2$ per μmole nicotinic acid. That a portion of the nicotinic acid is assimilated is further shown by the ability of nicotinic acid to serve as sole source of fixed carbon in growth experiments.

Pseudomonads from enrichment cultures containing tryptophan, anthranilic acid, nicotinic acid, or other carbon sources

The anomalous ability of the 'quinolinic' pseudomonad to oxidize vigorously some of the compounds which serve it as growth factors had no counterpart in any of twenty-nine other pseudomonads obtained from nicotinic acid enrichment cultures. Its auxotrophy is unique: none of twenty-one isolates from anthranilic acid (0.1 %) enrichments, nor any of another twenty-one isolates from DL-tryptophan (0.2 %) enrichments proved to be auxotrophic. This raised the question whether the present isolation technique is adequate for obtaining other specialized oxidative organisms. Den Dooren de Jong's (1947) studies with simple aliphatic amines, particularly diethylamine, provided a test case. No difficulty was experienced in the present experiments in obtaining these organisms with either 0.5 % diethylamine.HCl or 0.4 % ethylurea. On test, two strains each of *Protaminobacter rubrum* and *P. alboriflavum* thus isolated in the presence of growth-factor supplement were not auxotrophic. These enrichments yielded, however, a culture which gave rise to distinct red and white colonies which could not be grown apart from each other. Dr W. Vishniac has informed us that he has experienced a similar difficulty with diethylamine enrichment cultures.

Some of the pseudomonads isolated from cultures enriched with uracil (0.2 %) exhibited a requirement for aneurin.

DISCUSSION

Auxotrophy in pseudomonads

The isolation of the 'quinolinic' pseudomonad and aneurin-requiring pyrimidine oxidizers shows that auxotrophy in pseudomonads is not confined to photosynthetic forms. These experiments also indicate that an enrichment culture technique which provides growth factors, favours the isolation of a more varied representation of the organisms present in nature. Dr J. W. Foster has informed us of further examples of auxotrophy in this group: biotin is required for the growth of a pseudomonad capable of oxidizing lumichrome, and another which can oxidize riboflavin. West & Lochhead (1940) showed that many bacteria in the rhizosphere are auxotrophic; auxotrophic pseudomonads, it appears, may constitute a significant portion of the microflora in the rhizosphere.

None of the twenty-nine other cultures obtained by nicotinic acid enrichment was auxotrophic. The utilization as carbon sources of compounds in the anthranilic acid-tryptophan-nicotinic acid series may reflect valid taxonomic distinctions. Of the other twenty-nine nicotinic acid oxidizers, five oxidize tryptophan but not anthranilic acid, eight oxidize both tryptophan and nicotinic acid, three oxidize anthranilic acid but not tryptophan, and thirteen neither tryptophan nor anthranilic acid. Similarly, tryptophan-utilizing organisms and the anthranilic acid organisms showed all the possible combinations in the utilization of anthranilic acid, tryptophan and nicotinic acid.

The oxidation of nicotinic acid is not a general property of pseudomonads of the *Pseudomonas fluorescens* type. A strain of *Ps. pyocyanea* (*Ps. aeruginosa*) oxidized anthranilic acid and tryptophan but not nicotinic acid. Four strains of *Ps. fluorescens* supplied by Dr R. Y. Stanier, isolated, respectively, from benzoate, alanine, glycerol, and succinate enrichments, did not oxidize nicotinic acid; all four strains oxidized tryptophan and only one oxidized anthranilic acid.

The divergence in metabolic pathways underlying the observations that some tryptophan oxidizers utilize anthranilic acid and others do not, is being examined elsewhere (Stanier, Hayaishi & Tsuchida, 1951).

Quinolinic acid as a growth factor

The position of quinolinic acid in both protist and animal is problematic. Certain *Neurospora* mutants have a growth-factor requirement satisfied by nicotinic acid or by much higher concentrations of quinolinic acid (Henderson, 1949; Bonner & Yanofsky, 1949). Quinolinic acid corrects nicotinic acid deficiency in the rat but, again, at much higher concentrations than does nicotinic acid (Henderson, 1949). One explanation of the superiority of quinolinic acid as a growth factor for the 'quinolinic' pseudomonad is that quinolinic acid is in part converted to nicotinic acid and that the unconverted quinolinic acid blocks the subsequent oxidation of this nicotinic acid. Were this so, nicotinic acid might function more effectively as a vitamin in the

presence of quinolinic acid. However, quinolinic acid in concentrations 20 times that of nicotinic acid did not inhibit the oxidation of nicotinic acid. Experience in the use of the organism for the assay of quinolinic acid showed that the effect of the presence of nicotinic acid is additive—an indication that both function similarly. Work in progress at Yale indicates that resting suspensions produce measurable amounts of nicotinic acid from quinolinic acid. This suggests that the activity of quinolinic acid depends on its conversion to nicotinic acid, and that endogenously formed nicotinic acid may be less subject to oxidation than exogenous nicotinic acid.

The superiority of 3-hydroxyanthranilic acid over nicotinic acid as a growth factor may similarly depend on its conversion to nicotinic acid at a rate so slow as to allow the nicotinic acid in turn to be converted immediately to an active compound which is not subject to oxidation. Similarly, nicotinamide may be formed from nicotinic acid and not immediately subject to oxidation. Alternatively, the conversion of these compounds to nicotinic acid may be at a site in the organism which is somehow protected against oxidation.

The singular effectiveness of quinolinic acid as a growth factor for this organism stands out in a comparison with another Gram-negative bacterium, *Xanthomonas pruni*, whose growth-factor requirement is satisfied by all the known intermediates in the biosynthesis of nicotinic acid (Davis, Henderson & Powell, 1951). In the instance of *X. pruni*, however, quinolinic acid shows only one-hundredth the activity of nicotinic acid.

The information on the 'quinolinic' pseudomonad does not as yet settle the issue of whether quinolinic acid plays the role of a functional biosynthetic intermediate or that of a metabolic cul-de-sac.

Dr D. M. Bonner's interest in this investigation is gratefully acknowledged.

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