The Utilization of Pyridine by Micro-organisms

By F. W. MOORE

Department of Bacteriology, University of Birmingham

SUMMARY: Soil-enrichment cultures in a medium containing 0.1 % (v/v) pyridine as the sole organic compound gave plate counts of more than 1000 million organisms/ml. Organisms of the genus *Proactinomyces* were able to utilize pyridine, aniline, nicotinic acid, nitrobenzene, or phenol+ammonium ion as sole source of carbon, nitrogen and energy.

Very little is known of the decomposition by micro-organisms of simple cyclic compounds. The fact that such compounds do not accumulate in the soil indicates that degradation must occur. The chemical stability of such elementary six-membered ring compounds as benzene and pyridine is well known, and it is unlikely that any decomposition takes place in the soil as a result of purely chemical action. The occurrence of the pyridine ring in the form of nicotinic acid or its derivatives appears to be universal in living cells. Co-enzymes I and II each contain the nicotinamide moiety as an essential part of their molecules. The quantities of pyridine ring compounds which are returned to the soil on the death of animal and vegetable cells are extremely difficult to estimate, but it can be assumed that there is a steady supply of such compounds available for decomposition.

References to the dissimilation of pyridine by micro-organisms are few. Buddin (1914), when attempting to sterilize soil by the use of a pyridine solution, found that the pyridine was rapidly broken down by micro-organisms. Funchess (1917) showed that nitrification, using pyridine, occurred in soil; pyridine was almost as good a source of nitrogen for corn and oats as sodium nitrate. Robbins (1917) demonstrated that the bacterial content of soil rose rapidly after the addition of 0.1 % pyridine. He isolated a species of bacterium which was capable of growing in a medium containing pyridine as the sole source of nitrogen. den Dooren de Jong (1926) found that pyridine could be used as the sole source of nitrogen by *Aerobacter aerogenes*, *Serratia marcescens* and *Bacterium herbicola*. Ostroff & Henry (1939) reported the utilization of pyridine as a source of nitrogen by three species of marine bacteria. Koser & Baird (1944) studied the growth of bacteria on a medium which included nicotinic acid or closely related compounds. They found organisms of the *Pseudomonas fluorescens* group capable of using nicotinic acid as their sole source of carbon, nitrogen and energy. *Serratia marcescens* and related species were able to grow on a similar medium having $(\text{NH}_4)_2\text{HPO}_4$ as the nitrogen source. None of these organisms was able to grow when pyridine replaced the nicotinic acid.

Experimental methods

Medium. The basal culture medium used for most of the work was a modification of that used by Gray & Thornton (1928): K_2HPO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; NaCl, 0.1 g.; CaCl_2 , 0.1 g.; $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 , 1.0 g.; pyridine A.R.,

1.0 ml.; dist. water to 1000 ml. The pH was adjusted to 7.2–7.4, and the medium sterilized by autoclaving for 20 min. at 15 lb./sq.in. pressure. Solid medium was prepared by adding 400 ml. of 5 % agar (well-washed) to 600 ml. of the basal medium. Occasionally the composition of the medium was modified by the omission of the inorganic source of nitrogen, or by varying the percentage of pyridine.

Preliminary enrichment

Five g. of garden soil were shaken with 50 ml. of quarter-strength Ringer's solution. After standing for 5 min., 1 ml. of this suspension was added to 100 ml. of the basal medium, which included ammonium sulphate, and another 1 ml. was added to 100 ml. of the basal medium containing potassium nitrate. These liquid cultures were incubated at room temperature (15–20°) for 7 days.

Examination of stained and unstained preparations showed large numbers of non-motile, much-branched filamentous organisms, and smaller numbers of motile rods, non-motile cocci and Protozoa. These mixed cultures were subcultured by transferring one loopful of the liquid to 20 ml. of basal medium containing either $(\text{NH}_4)_2\text{SO}_4$ or KNO_3 . This subcultivation and enrichment was carried out four times, the cultures being incubated at room temperature for 5 days.

Isolation

Decimal dilutions to 1/10,000,000 of the final enrichment cultures were prepared in sterile quarter-strength Ringer's solution. Two drops (c. 0.1 ml.) of each dilution were spread over the surface of previously poured and surface dried plates of Lemco nutrient agar, which were incubated at room temperature for 10 days. Plates prepared from the highest dilutions (1/10,000,000) averaged 15–20 colonies/plate, indicating that the final enrichment cultures contained at least 1500 million organisms/ml. Isolated colonies were subcultured on Lemco agar slopes, and the resulting growths were again subcultured into the basal pyridine medium which included the same inorganic source of nitrogen as the enrichment culture from which the organism was isolated. Two more subcultures into the pyridine medium were carried out at 5-day intervals to minimize the carry-over of organic matter from the Lemco agar. Tubes showing visible growth were then subcultured into the basal medium without the inorganic nitrogen.

Identification

The organisms forming colonies on Lemco agar plates were classified by microscopical and cultural examination, as follows: *Proactinomyces*, c. 40 %; Gram-negative rods (mainly *Pseudomonas* sp.), c. 30 %; *Actinomyces*, c. 15 %; Gram-positive cocci and rods, c. 15 %.

When these organisms were inoculated in pure culture into the pyridine medium, almost every organism showed some growth in the first subculture. This was probably due to the fact that the initial inoculum was heavy and that a small quantity of organic matter was carried over. It was found that all the

Proactinomyces organisms were capable of continued growth in the basal pyridine medium, whereas none of the other organisms would grow in pure culture in it, even when an inorganic nitrogen compound was also present. The large number of organisms of the *Pseudomonas* type found in the final enrichment cultures suggests that, once the pyridine ring is broken by the *Proactinomyces* species, the pseudomonads are able to utilize the decomposition products as sources of carbon, nitrogen and energy.

There were two distinct groups of *Proactinomyces* present. The first group, which included about ten morphologically distinct types, was characterized by the production of a small, rather unstable, branched mycelium, which appeared

Table 1. *Effect of pyridine concentration on the growth of Proactinomyces*

The results were classified by visual examination into four degrees of turbidity: — no visible growth; + slight growth; ++ medium growth; +++ heavy growth.

Concentration of pyridine % (v/v)	Duration of incubation (days)						
	2	3	5	10	15	24	43
	Relative turbidity						
0.05	+	+	++	++	++	++	++
0.10	+	++	+++	+++	+++	+++	+++
0.15	+	++	+++	+++	+++	+++	+++
0.20	—	+	++	+++	+++	+++	+++
0.25	—	+	+	++	+++	+++	+++
0.30	—	—	+	++	+++	+++	+++
0.35	—	—	—	+	++	+++	+++
0.40	—	—	—	—	+	++	+++
0.45	—	—	—	—	+	+	++
0.50	—	—	—	—	—	+	+

granular when stained by Gram's method. Surface colonies on Lemco agar were pinkish, glistening and easily emulsified. A heavy sediment was produced in liquid culture. The second group, the various strains of which closely resembled one another, produced a relatively stable, much-branched and matted mycelium. Surface colonies were white, dull, waxy and difficult to emulsify. In liquid culture, a pellicle of the 'powdered chalk' type was produced, together with a flocculent growth which occasionally adhered to, and formed colonies on, the inner surface of the tube.

Growth in various concentrations of pyridine

The basal medium was modified by omitting the inorganic source of nitrogen, and by adding pyridine to give the following percentage (v/v) concentrations 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50. All the strains of organisms utilizing pyridine were subcultured from 4-day cultures in 0.10 % (v/v) pyridine medium into the liquid media of varied pyridine concentration. The results are shown in Table 1. All strains seemed to follow the same pattern, growth being more rapid in the lower concentrations and reaching a maximum after 5 days. As the concentration of pyridine was increased, the initial lag

period was extended. In the case of 0.50 % pyridine, no growth was visible for 21 days, and a maximum was not reached until 70–90 days.

The remainder of the investigation was carried out on two strains of *Proactinomyces*, one from each of the two groups mentioned earlier. The basal medium was modified by the substitution of other cyclic compounds in the place of pyridine. The concentrations used were 0.10 % (w/v) of phenol and of nicotinic acid, and 0.10 % (v/v) of aniline and of nitrobenzene. Both organisms were able to use the cyclic compound as the sole source of carbon, nitrogen and energy for their growth, except in the case of phenol, which was utilized when 0.1 % ammonium sulphate was added.

Some products of metabolism

Crystals formed in liquid cultures of each type of organism; these crystals gave positive reactions for calcium and oxalate. As the carbon:nitrogen ratio of pyridine is approximately 4:1, it is unlikely that the organisms assimilate all the nitrogen present in the medium. Microchemical tests for nitrate, nitrite and hydroxylamine gave uniformly negative results at all stages of growth. The progressive production of ammonia was demonstrated by the use of Nessler's reagent.

DISCUSSION

Previous evidence of the growth of pure cultures of micro-organisms in media containing unsubstituted six-membered ring compounds as their sole source of carbon and energy has been provided by Tausson (1929), who demonstrated that benzene was utilized by an organism which he called *Bact. benzoli*. Grant & ZoBell (1944), using absorption of oxygen as evidence of growth, reported that certain marine bacteria could utilize benzene.

The stability of unsubstituted aromatic compounds to microbial attack has been noted by many workers. ZoBell (1948) states that 'aromatic and naphthenic hydrocarbons having side-chains appear to be attacked more readily than similar homologues without side-chains'. Stanier (1948) found that benzene, toluene and xylene were not decomposed by fluorescent pseudomonads. The bacteria studied by Tausz & Peter (1919) failed to attack benzene.

On the other hand, reports of the breakdown of substituted cyclic compounds such as phenol, benzoic acid and nicotinic acid are more numerous (see Gray & Thornton, 1928; Stanier, 1948; and Koser & Baird, 1944). It appears that the substitution of one of the hydrogen atoms of the benzene or pyridine nucleus by a reactive group, such as —OH or —COOH, leads to a certain degree of susceptibility to microbial attack.

Pseudomonads and other Gram-negative rods have been shown to be capable of utilizing cyclic compounds as their sole source of carbon and energy by den Dooren de Jong (1926), Gray & Thornton (1928), Koser & Baird (1944) and Stanier (1948). It is interesting to note that although large numbers of motile Gram-negative rods were present in pyridine enrichment cultures, these organisms would not grow in pure culture in the pyridine media. Apparently *Proactinomyces* breaks down the pyridine molecule in such a way that the

products can satisfy the carbon, nitrogen and energy requirements of the Gram-negative rods as well as its own needs. Other organisms, reported by den Dooren de Jong (1926), Robbins (1917) and Ostroff & Henry (1939) to be capable of utilizing pyridine as a source of nitrogen and thus of rupturing the ring, could not satisfy their carbon and energy requirements with the remainder of the molecule, but needed an additional carbon compound, such as glucose. It appears from the evidence presented that, in pure culture, the utilization of pyridine as a sole source of carbon, nitrogen and energy is limited to organisms of the genus *Proactinomyces*, although certain other bacteria are able to grow in mixed culture with *Proactinomyces*, or to utilize pyridine as a source of nitrogen.

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