MICROBIAL METABOLISM OF CARBAMATES

II. NITRIFICATION OF URETHAN BY Streptomyces nitrificans

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The inhibition of hexokinase (Meyerhof and Wilson, 1948), choline oxidase (Goldfinger et al., 1947), and other enzyme systems by urethan and related carbamates has been studied extensively. Yet precisely how these compounds exert their physiological effects is unknown. Warburg (1921) considered urethan to be an "indifferent" narcotic, blocking dehydrogenases by nonspecific absorption on cellular interfaces. Inhibition of yeast respiration by urethan has been attributed to its behavior as a nonionic surface active agent (Lamanna and Campbell, 1953). This carbamate also inhibits the antibacterial action of low levels of sulfonamides (Johnson et al., 1943) and causes a decrease in the catalase content of Escherichia coli (Pontieri, 1953). The antagonism of the leucopenic and carcinogenic properties of urethan by pentose nucleotides (Cowen, 1949) and the reversal of urethan inhibition of E. coli by 2,6-diaminopurine (Skipper and Schnabel, 1952) suggest an interference with nucleic acid metabolism.

The over-all objective of the present studies was to compare the intermediary metabolism of microorganisms grown with and without carbamates as substrates. Such information, it is believed, may prove useful in elucidating the physiological mode of action of these compounds. For this purpose, cultures capable of developing on carbamates were isolated from various sources by suitable enrichment techniques (Schatz et al., 1954). This report deals with the unusual nitrifying ability of Streptomyces nitrificans, a heterotrophic organism that can utilize urethan as the sole source of nitrogen, carbon, and energy.

MATERIALS AND METHODS

The composition of the basal medium and conditions of growth for S. nitrificans have already been reported (Schatz et al., 1954). For Warburg studies, uniform cell material was produced in the liquid mineral base supplemented with 0.2 per cent urethan and 0.2 per cent Na₂ succinate·6H₂O. These cultures were aerated vigorously by bubbling washed and cotton-filtered air through the medium at a constant rate. After 5 to 7 days, yields of about 0.33 g dry weight of mycelium per liter were obtained. The growth was harvested by filtration through sintered glass, homogenized in a Waring blender with H/30 Sörensen's phosphate buffer at pH 7.0, rewashed with the buffer, diluted to the desired turbidity, and refrigerated until used. Single arm, 20 ml Warburg vessels, with the final liquid volume adjusted to 2.1 ml, were employed in a 30 C water bath. All flasks routinely contained air with KOH in the center well. The concentration of cell material per flask was 25.0 mm³ of wet packed mycelium, equivalent to 5.0 mg dry weight. Warburg experiments were carried out in the H/30 phosphate buffer at pH 7.0.

For studies on nitrite production by growing cultures, it was necessary to rule out contamination by nitrite which occurred in air, in many neutral and alkaline cp reagents, in the purest cotton available for plugging, in the highest grade filter paper, on Seitz filter pads, in aged distilled water, on air-dried glassware washed with tap water, and elsewhere. Therefore, all media were prepared with freshly distilled water and dispensed in pyrex flasks with glass-wool plugs. Before use, glassware was washed with sulfamic acid solution to destroy nitrite. The distilled water and other reagents employed were established to be free of nitrite as well as nitrate since the former can be derived from the latter by reduction. After autoclaving, the flasks were cooled in a desiccator containing air over sulfamic acid solution in order to remove any traces of nitrous anhydride. The flasks were inoculated then with spores suspended in nitrate and nitrite-
free basal medium and immediately replaced in the same desiccator which also contained the uninoculated controls.

Nitrite was determined by diazotization followed by colorimetry of the resultant red dye. The absence of nitrate was surmised from the constancy of nitrite concentration after subjecting an aliquot of the original cell-free supernatant to reduction by zinc dust. The identity of nitrite was established by its inability to diazotize after exposure to sulfamic acid and sodium azide which destroy nitrite.

RESULTS

One possible way of regarding urethan utilization is to view it as a means by which organisms remove a toxic compound. In this case, degrading the carbamate to ammonia and ethanol would yield products fortuitously serving as substrates. If this were so, organisms tolerating highest levels would be capable of metabolizing the compound. However, this was not true for a number of cultures tested (Schatz et al., 1954). E. coli, Bacillus subtilis, and Staphylococcus aureus grew in the presence of fairly high concentrations of urethan but failed to utilize it even as a nitrogen source in suitable glucose media.

Production of nitrite by S. nitrificans. Respirometry then was employed in an attempt to obtain information on how S. nitrificans utilized urethan. The results of a 10 hr experiment (figure 1) revealed that the consumption of oxygen in the presence of 50.0 µg of urethan, Q02 (mg) = 3.0, was twice as much as for the endogenously respiring cell material, Q02 (mg) = 1.5. Repeated experiments with cell material from the same culture and from different lots yielded similarly low values. Under the conditions employed, increased rates of respiration were not obtained even with very young cell material. However, the presence of urethan as substrate always resulted in an oxygen uptake over and above that of the endogenous respiration.

To test for conversion of urethan nitrogen to nitrite, the contents of the Warburg vessels, except for the KOH in the center well, were removed at the end of the experiment and centrifuged. The supernatants of both cell suspensions exposed to urethan contained 5.0 µg equivalent of NaN₃. This gave a very strong test, the limit of sensitivity being 0.01 µg. The endogenously respiring cell suspension and the urethan itself were nitrite-free. Neither the stock cell suspension nor the urethan solution contained nitrate. In several experiments of this kind, nitrite was always demonstrable after 5 to 10 hours only in vessels containing urethan as the respiration substrate. So far, final concentrations greater than 5.0 µg equivalent of NaN₃ per vessel have not been obtained.

Nitrite production from urethan also was observed in growing cultures (table 1). The nitrite found here was not carried over in the inoculum, nor could it have been derived from nitrate since nitrate was not present at the time of inoculation. The production of nitrite was of the same low order whether urethan was the sole substrate or was supplemented with succinate. Why the oxidation of organic nitrogen by the actinomycte does not continue to nitrate is perplexing since the organism readily reduces nitrate for cell synthesis. Perhaps the nitrite oxidizing enzyme may be more sensitive to oxygen than the enzyme involved in ammonia oxidation. If so, this would be the reverse of what has been reported for Pseudomonas aeruginosa (Sacks and Barker, 1949) and Hydrogenomonas facilis (Schatz and Bovell, 1952). In these bacteria, the transformation of nitrate to nitrite was more resistant to inactivation by oxygen than was the further reduction of nitrite.

Metabolism of urethan and other carbamates. The variation in physiological properties of carbamates with structural modification suggested testing derivatives of urethan for suitability as microbial substrates. With S. nitrificans it was found that in addition to urethan, growth occurred on n-propyl, n-butyl, and ethyl-n-
ethy carbamates as sources of nitrogen, carbon, and energy (table 2). However, n-methyl urethan allowed growth only in the presence of succinate. On these other carbamates as substrates, S. nitrificans also produced nitrite but no nitrate. At 0.15 per cent, ethyl-n-benzyl carbamate was not toxic but was not metabolized, while phenyl urethan was inhibitory.

Two other actinomycetes included in this study for comparison with S. nitrificans failed to develop on any carbamate as sole substrate. Streptomyces venezuelae grew only on urethan when succinate was also available. Mycobacterium smegmatis utilized urethan and three other carbamates but like S. venezuelae only in the presence of other carbon sources. When growing on carbamates, these organisms produced no nitrite or nitrate. With a soil inoculum, good bacterial growth occurred on all carbamates in a succinate medium.

**DISCUSSION**

The action of carcinogenic and anticancer compounds on microorganisms has attracted some attention (Tittler, 1948; Skipper, 1953), but the effect of microorganisms on these agents has been much less studied (ZoBell and Sisler, 1947). This neglect is surprising. As pointed out by Wolf (1952): "This problem of the mechanism of action of chemical carcinogens is closely linked..."
with the question what happens to the carcinogen in the organism. If we could gain some insight into the successive reactions undergone by the carcinogens to bring about malignancy, we could solve more than half of the problems of its mode of action."

As regards urethan, the assumption, tacit or overt, has been that this compound simply was hydrolyzed in vivo to ethanol, ammonia, and CO₂, or urea and CO₂, products which do not account for the physiological behavior of urethan. The studies of Skipper et al. (1948) and Boyland and Rhoden (1949) were concerned only with the production and fate of ethanol. The fact that S. nitrofasana produces some nitrite from urethan poses a question as to the possible in vivo production of organic nitrite and nitro compounds from urethan. The carcinogenicity of other carbamates (Larsen, 1948) raises the question of whether any pharmacological properties of carbamates are correlative with their microbiological utilization or conversion to nitrite.

Whether the pathway by which S. nitrofasana metabolises urethan involves an initial hydrolysis to ethanol, which occurs with certain soil organisms (Quastel and Scholefield, 1953), has not been established. Also unknown is the manner in which nitrite is formed. This may occur by way of direct oxidation of ammonia as in the classical autotrophic nitrifiers, or by derivation from oxime compounds (Jensen, 1951). Regardless of the mechanism involved, nitrification by the heterotrophic S. nitrofasana is unusual. Herefore, this ability generally has been considered to be characteristic only of Winogradsky’s obligately autotrophic bacteria. However, the production of the antibiotic chloramphenicol, a nitro compound, by Streptomyces lavendulae (Rebstock et al., 1949), and of β-nitropropionate (Bush et al., 1951) and nitrate (Schmidt, 1954) by Aspergillus flavus suggests that nitrification may be distributed more widely than has been supposed.

SUMMARY

Streptomyces nitrofasana has been shown to produce nitrite from urethan in Warburg experiments and from urethan as well as n-propyl, n-butyl, n-methyl, and ethyl-n-ethyl carbamates during growth. The rigid experimental conditions eliminated nitrite contamination from external sources and established that this heterotrophic nitrification does not proceed to the level of nitrate. The results are discussed with respect to mammalian metabolism of urethan and microbial nitrification.

REFERENCES


