MICROBIAL METABOLISM OF CARbamates

I. ISOLATION OF Streptomyces nitrificans, Spec. Nov., AND OTHER ORGANISMS WHICH GROW ON URETHAN

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The physiological properties of urethan (ethyl carbamate) are unexpectedly complex and bewildering for so simple a compound. It has long been recognized as a narcotic and a hypnotic (Goodman and Gilman, 1941). Because of its leucopenic action (Hawkins and Murphy, 1925) it temporarily alleviates human leukemia (Paterson et al., 1946) and myeloma (Loge and Rundles, 1949). It also inhibits the growth of transplanted sarcoma and dedifferentiates tumor cells (Hadow and Sexton, 1946). One of the few water-soluble carcinogens, urethan induces pulmonary carcinoma (Nettleship and Earle, 1943), an effect perhaps associated with its radiomimetic toxicity (Dustin, 1947) and production of chromosome breaks (Boyland and Rhoden, 1949).

The investigations reported here were undertaken in the hope that information on the microbial transformation of carbamates would contribute to the understanding of the physiology of these compounds. This paper describes the occurrence and isolation of microorganisms which utilize carbamates as substrates for growth.

MATERIALS AND METHODS

To isolate organisms capable of growing on urethan as the sole source of available carbon, energy, and nitrogen, the following distilled water basal medium supplemented with 0.2 per cent urethan was inoculated with over 50 samples of soils, muds, manures, and composts collected from different parts of the country: KH₂PO₄, 0.05 per cent; MgSO₄·7H₂O, 0.02 per cent; Fe as FeCl₃·6H₂O, 0.4 mg per cent; Ca as CaCO₃ plus minimum HCl to dissolve, 2.5 mg per cent; Mn as MnCl₂·4H₂O, 0.2 mg per cent; Zn as ZnSO₄·7H₂O, 0.04 mg per cent; Mo as Na₂MoO₄·2H₂O, 0.02 mg per cent; Cu as CuSO₄·5H₂O, 0.002 mg per cent; Co as CoCl₂·6H₂O, 0.02 mg per cent; ethylenediaminetetra-}

acetic acid as a nonmetabolizable complexing agent to increase availability of essential trace metals, 0.02 per cent; adjusted to pH 6.8 to 7.0 with NaOH.

Except where otherwise indicated, all cultures were stationary and were incubated aerobically at 28 to 30 °C in the dark. Serial transfers were made when growth became visible. Final isolation was accomplished by streaking on the agarized enrichment medium. The sole organism isolated was maintained on agar slants of the urethan medium. Other cultures that used carbamates merely as sources of nitrogen were isolated by means of the same 0.2 per cent urethan medium containing 0.2 per cent ethanol, 0.2 per cent Na₂ succinate·6H₂O, or 1.0 per cent glucose as additional substrate. All growth experiments were carried out with the same basal medium supplemented with carbamates or other compounds.

RESULTS

Isolation of Streptomyces nitrificans, sp. n.
Of the many enrichments with 0.2 per cent urethan as the sole source of nitrogen, carbon, and energy, a single culture was obtained from only one soil. This organism proved to be a hitherto undescribed actinomycete and is named Streptomyces nitrificans. According to the results of several experiments summarized below, this culture actually grew on urethan and not on possible decomposition products such as ethanol and ammonia, traces of which might be expected to form during steam sterilization. Whether autoclaved or filtered, given concentrations of the carbamate allowed the same amount of growth of the actinomycete and were equally inhibitive to urethan sensitive soil bacteria. Urethan resistant soil bacteria which utilized ethanol but not urethan failed to develop in an autoclaved car-
bamate medium containing ammonia but grew well upon addition of the alcohol.

Characteristics of *S. nitrificans*. On various simple and complex agar media, the urethan utilizing actinomycete grew better at 25 to 30°C than at 37°C and produced straight, branched, sporulated aerial hyphae. On blood agar, the organism developed a brick-red mycelium without hemolysis. On other media both solid and liquid, growth consisted of a gray sporulated surface with a pink to buff reverse. A well sporulated pellicle was formed in nutrient broth and other liquid media. On potato, carrot, and beet slants, growth was gnarled and wrinkled. The actinomycete was obligately aerobic, reduced nitrate to nitrite, slowly alkalinized milk without curdling, and hydrolyzed starch. It was non-acid-fast and did not liquefy gelatin, attack cellulose, produce indole, or form diffusible pigments in any of the simple or complex media tried.

*S. nitrificans* grew well on a variety of substrates other than urethan. With ammonia providing nitrogen in the basal medium, glucose, sucrose, mannitol, sorbitol, glycerol, ethanol, n-propanol, acetate, lactate, succinate, fumarate, and citrate permitted good growth. In a glucose containing medium, ammonia, nitrite, nitrate, urea, and guanidine were satisfactory sources of nitrogen. Several amino acids, purines, and miscellaneous other nitrogenous compounds supplied alone or with glucose in the basal medium supported growth.

Utilization of urethan as sole substrate was not an adaptive response. The organism grew as well on the carboxamate when first isolated from the urethan enrichment culture as it did after serial transfer over a two year period on various simple and complex media containing no urethan. In addition to its apparently unique ability to grow on urethan as sole substrate, this culture also produced nitrite from the carboxamate. Despite the fact that it developed with nitrate as nitrogen source, it did not oxidize the urethan nitrogen beyond nitrite. Since our search of the literature failed to reveal any actinomycete physiologically and morphologically similar, this organism was considered to be a new species and was named *S. nitrificans* by virtue of its unusual ability to nitrify.

Utilization of urethan by other microorganisms. To determine whether the 0.2 per cent concentration of urethan employed in the enrichments might have been inhibitive to some organisms, the following pure cultures selected at random as representing different types were inoculated into glucose-peptone media with and without 0.2 per cent urethan: *Escherichia coli*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptomycetes*, and *Mycobacterium smegmatis*. In an additional experiment, the urethan was replaced by *Nocardia*, *Streptomyces*, *Micromonospora*, *Corynebacterium*, *Serratia marcescens*, *Serratia marcescens*, *Mycobacterium smegmatis*, *Micromonaspora*, *Nocardia corallina*, *N. farrinica*, *N. gypseoides*, *N. gardneri*, *N. maculata*, *N. mexicana* (4 strains), *N. paraffina*, *N. polychromogenes*, *N. salmonicola*, 8 unidentified *Nocardia* spp., *Streptomycyes antibioticus*, *S. griseus*, *S. lavendulae*, 41 unidentified *Streptomycyes* spp., 4 unidentified molds. Since all 75 organisms grew equally well in the two media, it was concluded that, at 0.2 per cent, urethan had not exerted any significant selective effect in the original soil enrichments.

### TABLE 1

<table>
<thead>
<tr>
<th>ORGANISMS</th>
<th>MINERAL BASE PLUS*</th>
<th>MAXIMUM PER CENT URETHAN TOLERATED</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>DPM</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Chromobacterium violaceum</em></td>
<td>DPM</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>DPM</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>DPM</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>DPM</td>
<td>2.3</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>DPM</td>
<td>2.5</td>
</tr>
<tr>
<td>Soil inoculum</td>
<td>AD</td>
<td>4.0</td>
</tr>
<tr>
<td>Aerobic</td>
<td>AD</td>
<td>2.0</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>DGSU</td>
<td>1.25</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>DPM</td>
<td>1.75</td>
</tr>
<tr>
<td><em>Streptomyces venezuelae</em></td>
<td>AG</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Streptomyces nitrificans</em></td>
<td>P</td>
<td>2.0</td>
</tr>
<tr>
<td><em>8 unidentified soil actinomycetes</em></td>
<td>U</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>AG</td>
<td>1.8</td>
</tr>
<tr>
<td>(aerobic and anaerobic)</td>
<td>P</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Identified soil yeast</em></td>
<td>DPM</td>
<td>1.0–1.4</td>
</tr>
<tr>
<td><em>9 unidentified soil molds</em></td>
<td>DPM</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* D = glucose, 1.0 per cent; P = peptone, 0.5 per cent; M = molasses, 0.25 per cent; A = (NH₄)₂SO₄, 0.5 per cent; G = glycerol, 0.5 per cent; S = Na₂succinate-6H₂O, 0.2 per cent; U = urethan in the concentrations indicated.
This suggested a study of the urethan tolerance of bacteria, actinomycetes, and fungi. The results shown in table 1 strengthened the conclusion already drawn that inhibition by 0.2 per cent urethan had not been responsible for the sole isolation of S. nitrificans with the carbamate as the only substrate. The possibility that other urethan utilizing organisms might be obtained by means of a urethan enrichment medium supplemented with growth factors was considered. However, extensive tests of this kind yielded negative results.

Attempts to adapt organisms to tolerate high levels of urethan and then utilize urethan alone were unsuccessful. Some soil bacteria grew well in the basal medium containing urethan and ethanol or urethan and acetamide, a compound structurally similar to urethan, but did not multiply when subsequently transferred to the carbamate alone. Other soil bacteria which grew in the basal medium containing urethan, ammonia, and glucose failed to grow when inoculated into the sugar-free medium. However, numerous organisms made good growth on urethan as nitrogen source when glucose, ethanol, or succinate was also present. Among these were Mycobacterium enemagis, Streptomyces venezuelae, and various pseudomonads.

**DISCUSSION**

The isolation of S. nitrificans as the only organism capable of growing on urethan as the sole source of carbon, energy, and nitrogen was surprising. The low molecular weight of this compound, its structural similarity to ethanol and urea which are widely metabolized, its water-solubility, and its lack of toxicity at low levels would imply greater susceptibility to microbial attack. Moreover, the carbamate linkage is naturally occurring at least in physostigmine (eserine), and ammonium carbonate present in the soil solution is an equilibrium mixture containing ammonium carbamate (Karrer, 1946). For these reasons, urethan utilizing organisms were expected to be abundant in the soil microflora. This held true only for organisms which can grow in a glucose, ethanol, or succinate medium with urethan as the nitrogen source. Thus, the compound is not resistant to microbial attack; likewise, it is broken down readily by mammalian tissues (Skipper et al., 1948; Boyland and Rhoden, 1949).

Why organisms which grow on ethanol and ammonia as well as on ethanol and urethan will not grow on urethan alone is not known. Such cultures no doubt remove ammonia from urethan or else they could not grow with urethan as the nitrogen source. In addition, the resulting esters (ethyl bicarbonate or ethyl formate, depending on whether the urethan amide group is split off hydrolytically or reducibly) are compounds which can be expected to undergo spontaneous hydrolysis and thus liberate ethanol:

\[
\begin{align*}
\text{C}_2\text{H}_4\cdot\text{O} \cdot \text{CO} \cdot \text{NH}_2 + 2\text{H} &\rightarrow \text{C}_2\text{H}_4\cdot\text{O} \cdot \text{CO} \cdot \text{H} + \text{NH}_2 \text{NH}_2 + \text{C}_2\text{H}_4\cdot\text{O} \cdot \text{CO} \cdot \text{OH} \\
\text{C}_2\text{H}_4\text{OH} + \text{HCOOH} &\rightarrow \text{C}_2\text{H}_6\text{OH} + \text{H}_2\text{CO}_3
\end{align*}
\]

The isolation of S. nitrificans appears to have been largely fortuitous. Its presence in only one of more than 50 diverse source materials originally tested and the failure to isolate it again in repeated attempts during a period of seven years indicate a restricted distribution in nature.

**ACKNOWLEDGMENT**

These studies were initiated by one of us (A. S.) while with the Division of Experimental Chemotherapy of the Sloan-Kettering Institute for Cancer Research, New York City. The suggestions of Dr. C. Chester Stock of that institution and of Dr. S. H. Hutner of the Haskins Laboratories, New York City, during some of the early phases of this work, are gratefully acknowledged.

**SUMMARY**

Soil organisms capable of growing on urethan were isolated by the enrichment culture technique. One type, represented solely by the new species *Streptomyces nitrificans*, utilized urethan as the only source of carbon, energy, and nitrogen and produced nitrite during growth. A second group comprising numerous bacteria and actinomycetes utilized carbamates merely as sources of nitrogen and failed to grow on these compounds alone.

**REFERENCES**

Boylan, E., and Rhoden, E. 1949 The distribution of urethane in animal tissues by a