Technical Papers

Mechanism of Microbial Degradation of Nicotine

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As regards the biochemical degradation of nicotine we still lack knowledge, both of the products and of the mechanism of the reaction. Bucherer (*1*) reported the isolation of a purple crystalline substance from a cultural solution containing nicotine, after incubation with three species of *Bacterium*: *B. nicotianum, B. nicotinophagum,* and *B. nicotinovorum*; and Wenusch (*2*) found the *N*-methylmyosmine among the degradation products of nicotine. Neither of these two products was, however, satisfactorily characterized. Werle (*3*) studied the decomposition mechanism of nicotine, using livers of guinea pigs, and considered that nicotine probably might be converted first to *N*-methylmyosmine, then to nicotyrin, and further degraded.

We isolated from soil a species of bacteria which probably belongs to *Pseudomonas* and which can utilize nicotine as a carbon as well as a nitrogen source. Using this microorganism we obtained the following results.

The bacterium was cultured at 30° C in a medium containing nicotine (0.2%) (I); KH_2PO_4 (0.1%); $MgSO_4$ (0.05%); $FeSO_4$ (0.3 ml 1% solution/liter); $CaCl_2$ (0.3 ml 1% solution/liter); and $MnSO_4$ (trace). The pH of the solution was adjusted to 6.0, and it was aerated with the aid of an aspirator. After 24 hr incubation the pH value suddenly declined to about 4.6 and the cultural solution abruptly became turbid. Keeping pace with this, the quantity of nicotine diminished rapidly. After this stage the pH value changed little, if at all, and the velocity of consumption of nicotine in the solution diminished.

After 5 days' incubation at 30° C, a 5-liter solution (pH 4.6) was filtered through a celite layer, and the filtrate was evaporated under reduced pressure to about 500 ml. From the ether extract of this condensate we isolated colorless needles (IV) that melted at 161°–162° C. The mother liquor thus treated was then evaporated again to about 100 ml, and from it a purplish-gray powder (VI), which melted at 315° C, was precipitated. The pH of the solution from which substance VI was then filtrated was increased to 8 by the addition of solution of sodium hydroxide. When an ether extract of this solution was evaporated, unchanged nicotine was found. The solution was then made strongly alkaline and again extracted with ether. After the ethereal solution was evaporated, the residual oil was distilled *in vacuo*. The distillate (III), bp 93.5° C (1.5 mm), formed a picrate melting at 127°–130° C after recrystallization from water. When the strong alkaline solution was then acidified and a saturated aqueous solution of picric acid was added to it, a picrate (V) was obtained that decomposed at 316° C.

When dissolved in water, compound IV, which was obtained in colorless needles of mp 161°–162° C, gave a solution of pH 4.4, and when a small quantity was added to a sodium bicarbonate solution the evolution of carbon dioxide was observed, suggesting its acidic nature. The substance gave a strong 2,4-dinitrochlorobenzene reaction, showing a pyridine nucleus, and gave a red coloration with sodium nitroprusside reagent, showing the presence of a carbonyl group. (Anal: Calcd for $C_9H_9O_3N$: C, 60.31; H, 5.02; N, 7.82. Found: C, 60.08; H, 5.00; N, 7.44.) It also gave a semicarbazone which decomposed at 202°–203° C. Compound IV gave nicotinic acid (mp 232° C) when oxidized with potassium permanganate.

When substance IV was fused with caustic potash and the resulting mixture was dissolved in water and extracted with ether, the ethereal solution gave almost nothing, even when extracted after acidifying. The acidified solution gave, however, a picrate which melted at over 300° C after recrystallization from 60% methanol. (Anal: Found: C, 27.50; H, 1.44; N, 17.03.) From these properties this compound was identified as the decomposition product V.

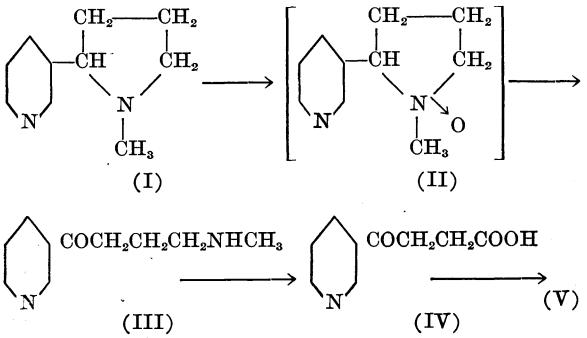
The picrate of III melted at 127°–130° C when recrystallized from water. (Anal: Calcd for $C_{22}H_{20}O_{15}N_8$: C, 41.51; H, 3.15; N, 17.61. Found: C, 41.72; H, 3.38; N, 18.51.) When recrystallized from absolute methanol it had a mp of 154°–156° C. The ultraviolet absorption spectrum of III showed maxima at 223 mμ and 263 mμ and minima at 211 mμ and 241 mμ, respectively.

Haines and Eisner (*4*) identified pseudo oxynicotine, first described by Pinner and Wolfenstein (*5*), as 3-pyridyl-3-methyl-aminopropyl ketone. The latter workers prepared this compound by heating oxynicotine (II) with hydrochloric acid in a sealed tube at 140° C. Haines and Eisner further reported on the conversion of pseudo oxynicotine to *N*-methylmyosmine. From their results and also our own, it seems likely that substance III and pseudo oxynicotine are the same, because the characteristics of substance III agree well with those of pseudo oxynicotine. As Haines and Eisner described, the picrate of pseudo oxynicotine melts at 128°–130° C (dipicrate of pseudo oxynicotine) when recrystallized from water and at 158°–160° C (dipicrate of *N*-methylmysomine) when recrystallized from absolute methanol. The ultraviolet absorption spectrum of pseudo oxynicotine showed maxima at 223 mμ and 263 mμ and minima at 211 mμ and 241 mμ, respectively.

Substance IV, which showed the properties of both a weak acid and a ketone, may have the molecular

formula of $C_9H_9O_3N$ and may be derived from pseudo oxynicotine by oxidative activities of the microorganism. This acid seems to be 3-succinoyl pyridine, and its constitution is now being studied by synthesis from nicotinic acid.

From the above-mentioned results we may now assume the mechanism of the decomposition of nicotine by the microorganism as follows:



Substance VI has almost the same characteristics as the compound obtained by Bucherer. (Anal: Found: C, 55.40; H, 4.65; N, 7.68.) Substance V, which may be produced through further decomposition from IV, is not extracted with ether from the acidic or alkaline solution, but from the acidified solution substance V is precipitated as picrate, mp 316° C, when recrystallized from 60% methanol (Anal: Found: C, 27.29; H, 1.34; N, 16.77.)

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Influence of Age of Serum on Microbiological Response[1]

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During investigations in this laboratory involving microbiological assays of blood from patients with malignant neoplastic diseases, it has been observed that storage of serum significantly influences the degree of response obtained with certain lactic acid organisms.

The basal medium contained 2% glucose and salts as employed by Dunn *et al.* (*1*), but 0.03%–0.05% Difco yeast extract was added in place of the growth factors and amino acids used by these workers. In the absence of yeast extract, significant growth response to serum did not occur. With a yeast extract concentration of 0.03%–0.05%, satisfactory responses to serum were obtained, with minimal growth in the control medium without serum. The nature of the component(s) of serum responsible for the observed stimulation of growth of microorganisms is unknown and, in fact, was not a primary concern of the studies reported here.

Double-strength medium was brought to boiling, filtered, and autoclaved for 15–20 min at 15 psi. Cultures were carried on Difco micro inoculum agar and were usually transferred twice in micro inoculum broth before use. Cells were washed once with distilled water, and 1–2 drops of a 1:100 dilution were used as inoculum. Incubation was at 37° C. Sera were obtained from prisoners at the state penitentiary in Huntsville, Texas, and from employees and patients in this hospital. All sera were presumably fasting. Hemolyzed sera were discarded, since it was noted that such sera yielded abnormally high responses. Growth was estimated, generally from triplicate tubes, in terms of optical density (log L_0/L) calculated from Coleman spectrophotometer readings at 590 μ. Serum concentrations employed ranged up to 25%.

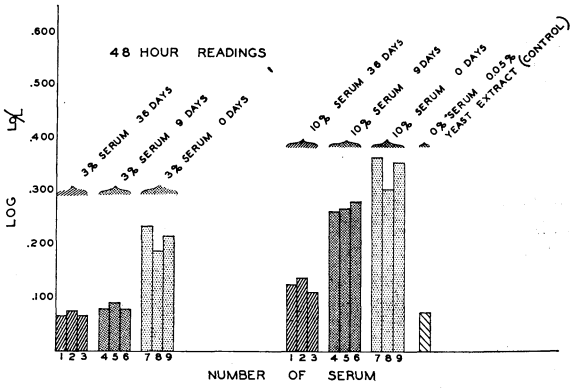

FIG. 1. Variation in response of *L. casei* Texas with age of normal sera.

Three groups of 3 sera each stored at 4° C for 0, 9, and 36 days, respectively, were tested simultaneously at two concentrations with *Lactobacillus casei* Texas (Fig. 1). Two control experiments, one with 10 fresh normal sera and the other with 5 fresh normal sera and 5 fresh sera from cancer patients, failed to show variations approaching the magnitude of those shown in Fig. 1. Responses to sera from patients with malignant neoplastic disease were similar to those obtained with normal sera.

Six pools of sera collected from five volunteers at varying times over a period of a month were tested simultaneously at a concentration of 10% with five organisms. A marked decrease in response of *L. casei* Texas occurred with increasing age of serum, whereas responses of *Lactobacillus brevis* ATCC 8287 and

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