Microbial Degradation of Nicotine III. The γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric Acid Intermediate

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Introduction

Tobuchi (13), Wada (9), Frankenburg & Vaitekunas (1), and Hylin (6) reported the presence of 3-succinoyl-6-hydroxypyridine as an intermediate in the bacterial degradation of nicotine after three or more days of incubation. A similar compound was detected when some of the bacterial isolates which we have described previously (3, 4) were incubated for sixteen hours.

The media used by the above-mentioned investigators in culturing their bacteria were found, by comparison, to be noticeably deficient and this fact probably necessitated the prolonged incubation periods. Such extensions of reaction times may obscure the sequential pattern of nicotine degradation by a final accumulation of intermediates of varying metabolic significance. Suboptimal environmental conditions which cause delayed growth may bear upon not only the rate but also the pattern of nicotine degradation.

This paper is concerned with the formation, isolation and metabolism of one intermediate of nicotine degradation formed during the first twelve hours of incubation by one of our non-blue-pigment-forming isolates.

Materials and Methods

Nicotine-degrading bacterial isolates Nos. 1 through 6 were grown in/on Sguros' medium containing 4.0 g of nicotine per liter of medium. The maintenance of the cultures, preparation of inocula, incubation conditions, and manometric technique used were described previously (3, 4). The medium was distributed in 100 ml aliquots into 300 ml baffled Erlenmeyer flasks, sterilized at 121°C for fifteen minutes, inoculated by resuspended centrifuged cells, and incubated on a rotary shaker operating at 250 rpm on a one inch diameter eccentric at room temperature.

Changes of ultraviolet absorption spectra were followed with a Beckman DK_2 spectrophotometer. Nicotine, obtained from Distillation Products Industries, was determined in samples by a local modification of Willit's procedure (14). Infrared analysis was carried out with a Perkin-Elmer Infracord Model 137 using thin films deposited on silver chloride discs. The thin layer chromatography procedures have been described by Stahl (9).

Results and Discussion

Chicago tap water used in the preparation of Sguros' medium was found to accelerate bacterial growth as well as the rate of nicotine degradation (4). The activities of isolates Nos. 3, 5, 6 and, to a smaller extent, No. 2, resulted in the appearance of ultraviolet absorption maxima at 275 mμ in an acidic medium after sixteen hours of incubation. Blue pigment-forming isolates Nos. 1 and 4 only slightly broadened the original nicotine maximum in the direction of longer wavelengths. Culture No. 3 was selected for the isolation of the intermediate which appeared similar to that obtained by the above-mentioned investigators after prolonged incubation periods (1, 6, 9, 13). They referred to this intermediate, partly for reasons of historical precedent and convenience, as 3-succinoyl-6-hydroxypyridine.

None or negligible amounts of distillable nicotine were found repeatedly after twelve hours of incubation using sixteen to twenty-four hour old inoculum. During this incubation period, an initial inoculum of approximately 10 mg of dry cellular weight per 100 ml of medium increased about tenfold and the pH became slightly acidic (6.2). In 0.1 *N* NaOH, a single well-defined ultraviolet absorption maximum appeared at 301 mμ. Within a subsequent twelve hours of incubation, this peak gradually faded out and no other absorption developed within the 200 to 340 mμ range.

Cellular suspensions, after twelve hours of incubation, were filtered through a one-half inch bed of Celite filter aid and the filtrate was concentrated under partial vacuum at 55°C in a nitrogen atmosphere. With the volume reduced to about one twentieth of the original, the concentrate was acidified with HCl to pH 2.0 and the resulting precipitate was filtered and air dried. Ultraviolet absorption spectra of the initial suspension, the filtered broth, the concentrate, and the recovered solid material were identical.

The recovered solid was an off

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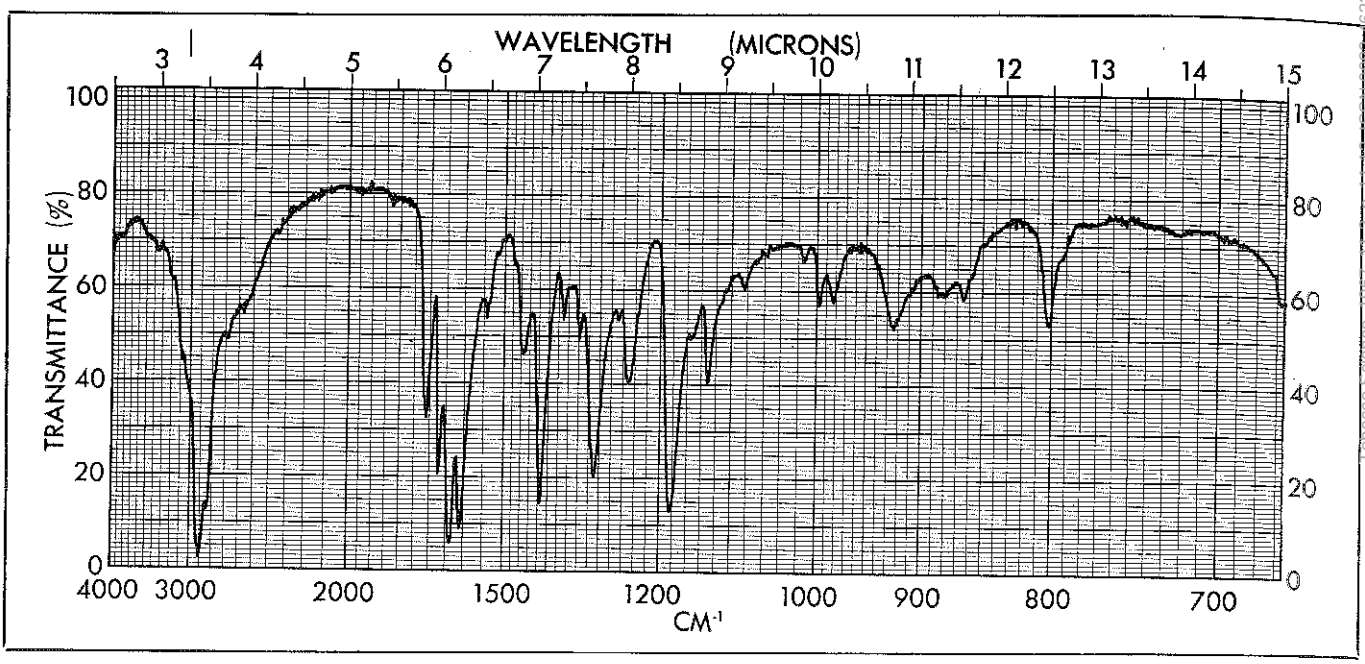

Figure 1. The infrared spectrum of γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid Nujol mull.

white powder having a melting point of 288° to 289°C and the following ultraviolet absorption maxima and minima:

	Max.	Min.
In acidic medium (0.001 *N* H_2SO_4)	275 mμ	240 mμ
In alkaline medium (0.01 *N* NaOH)	301 mμ	240 mμ

In one instance, 2.75 g (31.7% of theory) was obtained from 1.8 liter of filtered bacterial suspension. The intermediate was only slightly soluble in water at pH 4.4. It was insoluble in conventional solvents, but soluble in slightly alkaline water. The infrared spectrum shown in **Figure 1** resembles closely the one attributed to 3-succinoyl-pyridone-6 by Wada et al (10).

The elemental analysis compatible with the formula $C_9H_9NO_4$ was as follows:

	Found %	Calculated %
C	54.35	55.40
H	4.75	4.62
N	7.22	7.18
O (by diff.)	33.68	32.80

Different levels of inocula were used to obtain bacterial suspensions of varied progressions. With the same 10 mg of dry cellular weight of inoculum per 100 ml of medium, ultraviolet absorption maxima of 263 and 220 mμ in acidic medium were observed as early as four hours after inoculation. Subsequently, the absorption peak became broader and finally a clearly defined maximum at 275 mμ appeared after twelve hours of incubation. With smaller inocula, the absorption maxima at 263 and 220 mμ remained for twelve and sometimes twenty-four hours before occurrence of the shift toward 275 mμ. With larger inocula, the shift from the nicotine maximum at 259 mμ to 275 mμ was gradual without clear definition of the maxima at 265 and 220 mμ. The fading out of an intermediate showing an absorption maximum at 275 mμ began before the twelfth hour of incubation with no new maxima appearing.

The intermediate with ultraviolet absorption maxima at 263 and 220 mμ in acidic medium, characteristic of pseudooxynicotine (12), was obtained from an alkaline ether extract of twenty-four-hour-old suspension inoculated with less than 10 mg of dry cellular weight equivalent of isolate No. 3. It was of oily consistency and its picrate melted at 123°-126° C. Using two systems of thin layer chromatography, R_f values also corresponded to that of pseudooxynicotine (8). Thus, it appears that pseudooxynicotine is a direct precursor of γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid in the nicotine degradation pattern of the fast-growing non-blue-pigment-forming isolate No. 3.

When the isolated γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid was used as a substrate, washed sixteen to twenty-four-hour-old cells of isolate No. 3 were able to utilize it readily and without any lag period. After two hours and with an excess of substrate, the contents of the Warburg manometric vessels were examined spectrophotometrically. In basic solution, only varying levels of absorption at 301 mμ were observed. When the reaction time was prolonged until only a small O_2/CO_2 exchange was recorded, the contents of the flasks showed no ultraviolet absorption between 220 and 340 mμ.

Isolates Nos. 5, 6 and, to a smaller extent, No. 2, also utilized the γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid readily as indicated by no delay in oxygen uptake and carbon dioxide formation using the Warburg apparatus. Isolates Nos. 1 and 4 did not oxidize the intermediate to any significant extent. As pointed out previously, the ultraviolet absorption spectrum of the acid was not detected when periodic samples of these two cultures were examined spectrophotometrically.

A similarity appears between the inability of the two blue pigment-forming isolates to utilize γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid and the pathway of bacterial nicotine oxidation recently postulated by Rittenberg et al (2). The culture which he used also formed blue pigment and his scheme did not include this intermediate. The proposed pathway of nicotine oxidation includes 6-hydroxypseudooxynicotine and the ultraviolet absorption maximum (5) of this substance was noted during the incubation cycles of isolates Nos. 1 and 4. As reported previously, these two isolates degrade nicotine at a slower rate than those which do not form blue pigment (3).

The isolates used in the manometric experiments were grown in Sguros' medium prepared with Lancaster water. This indicated that

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the components of Chicago water were not needed by resting cells to bring about the degradation of an intermediate which they formed rapidly in the early stages of incubation. This is considered to indicate that the components of Chicago water affected the rate of formation of this intermediate and not the pattern of nicotine degradation for isolates Nos. 2, 3, 5 and 6. It is also in agreement with Stanier's concept of simultaneous adaptation when an intermediate is utilized readily by cells grown in the presence of the original substrate which, in this case, was nicotine (7).

With about a tenfold increase of dry cellular weight in twelve hours of incubation, some of the nicotine had to contribute to the growth of cells within a very short time. The medium contained 100 mg of yeast extract which corresponded to about 8 mg of nitrogen. The nitrogen content of the dried cells was found to be 10 to 11%, and this corresponds to 100 to 110 mg of cellular nitrogen per liter of bacterial suspension after twelve hours of incubation. With a nicotine nitrogen content of 692 mg/liter of medium, it appears that some of the nitrogen was used within the first two hours at which time light transmittance measurements indicated an increase in the number of cells. Whether the same scheme operates at an accelerated rate or a completely different one is available to acquire needed energy and the components for cellular growth remains to be established.

Hylin (6) found γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid to be a terminal nonmetabolizable compound formed only by mature cells. In our instance, growing twelve-hour-old cells as well as mature twenty-four-hour-old cells of non-blue-pigment-forming isolates utilized the intermediate readily. The results support the postulations of Tobuchi (13), Frankenburg & Vaitekunas (1), and Wada (9) who reported that it was oxidized further to yield aliphatic compounds. Tobuchi and Wada found it necessary to grow their nicotine-degrading bacteria for four to five days before individual fragments of nicotine degradation were isolated and identified; the reported purple color of Wada's isolated intermediate is in contrast to either the colorless or white preparations of others. Frankenburg & Vaitekunas, as pointed out by Wada, did not give any details concerning the experimental conditions and time periods used.

Since some of the intermediates of nicotine degradation are unstable (11), it may be speculative to attribute a metabolic significance to compounds which can be found after prolonged incubation periods. Some of them well may be the products of decomposition and autoxidation of other nicotine intermediates. This could be minimized by more suitable conditions — namely, less deficient media — which would rednce effectively the reaction periods.

Soil, which is likely to be the original habitat of nicotine-degrading bacteria, is much more generous in supplying an optimal environment (including nutritional requirements) than an oversimplified mixture of several mineral constituents dissolved in distilled water.

Summary

Under conditions supporting rapid growth, γ-[6-hydroxy-(3-pyridyl)]-γ-oxobutyric acid appeared to be the last major pyridine-containing intermediate formed within twelve hours by the non-blue-pigment-forming nicotine-degrading bacterial isolate No. 3. When isolated, it was readily oxidized without any time lag by other non-blue-pigment-forming cultures. The rate of its formation was enhanced noticeably by as yet unidentified components of Chicago tap water. Pseudooxynicotine appeared to be the intermediate preceding its formation.

The isolates which formed blue pigment were unable to oxidize it and there was no iudication of its formation during their incubation cycle.

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