THE ABSENCE OF A TRYPTOPHAN-NIACIN RELATIONSHIP IN ESCHERICHIA COLI AND BACILLUS SUBTILIS1, 2

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Nutritional studies with mammals led to the first suggestion of a relationship between tryptophan and niacin (Krehl et al., 1945). Subsequent investigations have established tryptophan as a precursor of niacin in many organisms and have led to the identification of several of the intermediates in this biosynthetic pathway (for reviews, see Dalgliesh, 1951; Bonner and Yanofsky, 1951). Direct demonstrations of the conversion of tryptophan to niacin have been provided by tracer studies performed with mammals (Heidelberger et al., 1949) and the mold Neurospora crassa (Partridge et al., 1952). In the latter case it was also shown that in this organism all niacin synthesis proceeds from tryptophan.

Comparatively little is known about niacin synthesis in bacteria. Marnay (1951) has concluded, on the basis of inhibition studies, that the pathway of niacin synthesis in Escherichia coli is the same as in Neurospora. Ellinger and Abdel Kader (1949) have also investigated niacin synthesis in E. coli and have presented evidence suggesting that tryptophan does not serve as a niacin precursor but functions catalytically in its formation. Several bacterial species can carry out a key sequence in niacin synthesis from tryptophan, the conversion of tryptophan to kynurenine (Stanier et al., 1951; Tabone and Robert, 1952). The conversion of kynurenine to niacin, however, has only been shown in the bacterium Xanthomonas pruni (Davis et al., 1951). This organism also appears to be capable of forming niacin from tryptophan.

During investigations with niacin auxotrophs of E. coli and Bacillus subtilis it became apparent that intermediates in niacin synthesis in Neurospora would not support the growth of the bacterial mutants. This observation and others suggested the possibility that E. coli and B. subtilis synthesize niacin by a different pathway from the one operative in Neurospora. Experiments were therefore carried out to determine if tryptophan serves as a precursor of niacin in E. coli and B. subtilis. The results of these experiments are presented in this paper.

MATERIALS AND METHODS

The tryptophan auxotrophs of E. coli and B. subtilis, employed in this investigation, and the compounds which support their growth are listed in table 1. In addition to the tryptophan auxotrophs, three niacin auxotrophs of E. coli and eight niacin auxotrophs of B. subtilis were also studied. The E. coli auxotrophs were isolated from ultraviolet irradiated populations of the K-12 strain using the Adelberg and Myers (1953) modification of the penicillin selection method (Lederberg and Zinder, 1948; Davis, 1948). The B. subtilis (Marburg strain) auxotrophs were kindly supplied by Dr. P. R. Burkholder. The composition of the minimal medium used in experiments with E. coli has been given previously (Rickenberg et al., 1953). The minimal medium described by Monod (1942) was used in experiments with B. subtilis. Niacin was determined by a modification of a microbiological method employing Lactobacillus arabinosus (Association of Vitamin Chemists, Inc., 1947). Indole was assayed colorimetrically with Ehrlich’s reagent. Tryptophan was determined microbiologically with strain T-1. The minimal medium was supplemented with 0.5 per cent acid hydrolyzed casein in assays with this strain. Counting was done with a gas flow counter in the Geiger region. The C14 uniformly labeled glucose was purchased from Isotopes Specialties Corporation and was diluted with unlabeled glucose to 6 × 106 cpm/mg carbon.

Labeled indole could not be counted directly because of its volatility. To overcome this difficulty indole was converted to a nonvolatile...
Response of tryptophan auxotrophs of Escherichia coli and Bacillus subtilis to anthranilic acid, indole, and tryptophan

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Supplement</th>
<th>Anthranilic Acid</th>
<th>Indole</th>
<th>Tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T-1</td>
<td></td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>T-2</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>T-3</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>168</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>156</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = compound supports growth; – = compound does not support growth.

Product, tryptophan, with a partially purified tryptophan synthetase preparation (Yanofsky, 1952) from Neurospora crassa.

EXPERIMENTAL RESULTS

Preparation of labeled indole and tryptophan, and isolation of C14 uniformly labeled indole.

Strain T-1 was grown in 200 ml of minimal medium supplemented with 600 μg of L-tryptophan and 300 mg of uniformly labeled glucose. After incubation for 48 hours at 37 C the cells were removed by centrifugation and the supernatant solution (adjusted to pH 9) concentrated in vacuo until it gave a negative test for indole. The distillate, containing the indole, was made slightly alkaline and was extracted with several portions of ether. The ether solution contained 4.6 mg of indole (assayed colorimetrically). Indole (41.4 mg) was added to the ether solution which was then dried overnight over Na2SO4. The ether solution was then taken to dryness in vacuo and the residue dissolved in a minimum quantity of warm water. The crystals of indole which appeared on cooling were filtered off and recrystallized from water. The indole obtained gave 4.92 × 10^6 cpm/mg (5.99 × 10^4 cpm/mg carbon) when counted as described previously. Since the cpm/mg carbon of the indole isolated (correcting for dilution) and the uniformly labeled glucose fed were essentially the same, the indole can be assumed to be uniformly labeled.

More recently the picrate derivative of indole was prepared and counted. 5.1 × 10^4 cpm/mg indole were obtained by this method.

Conversion of labeled indole to tryptophan. Five tubes, each containing 2.5 ml of a solution of C14-indole (1 mg), 12 mg of L-serine, 1 ml of a solution of diammonium pyridoxal phosphate (200 μg), 0.2 ml of 5 × 10^-3 M glutathione, 0.8 ml of 0.5 M phosphate buffer at pH 7.8, and 2 ml of a partially purified Neurospora tryptophan synthetase preparation in a total volume of 10 ml, were incubated at 37 C for 1½ hours. At the end of this period the incubation mixture in each tube gave a negative test for indole. The contents of the five tubes were pooled and deproteinized with 30 ml of 0.5 % perchloric acid. The precipitate was washed once with 10 ml of 0.25 % perchloric acid and the washings added to the deproteinized solution. This solution, after neutralization, was used as a source of C14 labeled tryptophan. The tryptophan content of the solution was 79 μg/ml. The niacin content was 0.6 μg/ml. The tryptophan content of the supernatant solution from a control experiment from which indole was omitted was less than 1 μg/ml.

Growth tests with niacin auxotrophs. A group of compounds, including several of the known intermediates in niacin synthesis from tryptophan, was tested for the ability to support the growth of three niacin auxotrophs of E. coli and eight niacin auxotrophs of B subtilis. The compounds tested were: anthranilic acid, indole, tryptophan, kynurenine, 3-hydroxykynurenine (kindly supplied by Dr. A. Butenandt), 3-hydroxyanthranilic acid, quinolinic acid, kynurenic acid, xanthurenic acid (kindly supplied by Dr. W. Lepkovsky and Dr. R. Shayer), and ornithine. Of these, only quinolinic acid supported growth. However, the response to quinolinic acid could be accounted for by the niacin content (0.047 per cent by assay with L. arabinose) of the quinolinic acid sample which was used. Thus, none of the compounds tested appears to satisfy the niacin requirement of the auxotrophs examined.

Inability of tryptophan auxotrophs to convert kynurenine to anthranilic acid. The kynureninase of neurospora (Jakoby and Bonner, 1953) and of mammals (Dalgliesh et al., 1951) has been shown to carry out the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. This reaction would appear to be an essential step in niacin synthesis in these organisms. Kynureninase also catalyzes the conversion of kynurenine to an-
Anthranilic acid. This latter reaction accounts for the ability of kynurenine to support the growth of anthranilic acid–responding tryptophan auxotrophs of neurepsora. Kynurenine should also support the growth of similar tryptophan auxotrophs of E. coli and B. subtilis if kynureninase is present and functional in these organisms. The results of an experiment designed to examine this point are presented in table 2. It can be seen that neither tryptophan auxotroph could use kynurenine for growth. This finding suggests that the strains examined lack kynureninase activity. This observation is consistent with the view that these strains are unable to convert tryptophan to niacin.

**Growth of strain T-2 on labeled tryptophan.** As a test of the conversion of tryptophan to niacin, tryptophan auxotroph T-2 was grown in minimal medium supplemented with labeled tryptophan, and the radioactivity of the niacin synthesized determined. A flask containing 750 ml of minimal medium (0.5% per cent glucose as carbon source) supplemented with 40 ml of the labeled tryptophan preparation was inoculated with strain T-2. After incubation for 16 hours at 37°C the cells were removed by centrifugation. The supernatant solution was concentrated in vacuo to about 20 ml and enough concentrated HSO₄ added to make the solution approximately 6 N. The cells were suspended in 30 ml of 6 N HSO₄. Both fractions were autoclaved for 40 minutes at 15 lb pressure. The hydrolysates were combined and centrifuged to remove insoluble material. The supernatant solution contained 255 µg of niacin.

**Isolation of niacin.** The first step in the isolation procedure involved adsorption of the niacin on Lloyd’s reagent. Four grams of Lloyd’s reagent were added to the supernatant solution and the mixture stirred for 5 minutes. The Lloyd’s reagent was removed by centrifugation, and an additional 3 g were added to the supernatant solution. After centrifugation, the supernatant solution, which was practically niacin-free, was discarded. The Lloyd’s reagent was washed twice with 20 ml portions of boiling water. Niacin was eluted from the Lloyd’s reagent by stirring with four 20 ml portions of hot 5 N NH₄OH. The ammonia eluates were combined and concentrated in vacuo to a few ml. Approximately 3 volumes of absolute ethanol were added to the concentrate and the mixture placed at 2°C for 3-4 hours. The precipitate which formed was removed by centrifugation and the supernatant solution concentrated in vacuo to a few ml. This whole fraction was applied to a sheet of Whatman no. 3 filter paper with a modified kymograph (Yanofsky et al., 1950) and chromatographed using the ascending method (Williams and Kirby, 1948) (the developing solvent contained 25 ml butyl alcohol, 50 ml propyl alcohol, 25 ml of water, and 1 ml of 1 N NH₄OH). The niacin band of the chromatogram was located using a pour plate seeded with L. arabinosus, as described elsewhere (Bonner and Wasserman, 1950). The niacin band was cut out and eluted with hot ethanol. The eluate was concentrated in vacuo and rechromatographed (the developing solvent contained 75 ml butyl alcohol, 15 ml formic acid, and 10 ml of water). The niacin band was located and eluted as before and concentrated in vacuo to a few ml. The concentrate was diluted to 10 ml, and 0.2 ml was removed and assayed for niacin. The concentrate contained 52 µg of niacin. Niacin (5.148 mg) was dissolved in the solution. The solution was taken to dryness, the niacin sublimed in vacuo, and the sublimate crystallized

<table>
<thead>
<tr>
<th>Additions</th>
<th>Klett Reading</th>
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<tr>
<td></td>
<td>Strain T-3</td>
</tr>
<tr>
<td>Anthranilic acid 3 µg</td>
<td>33</td>
</tr>
<tr>
<td>Anthranilic acid 5 µg</td>
<td>38</td>
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<td>Anthranilic acid 10 µg</td>
<td>67</td>
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<tr>
<td>Anthranilic acid 20 µg</td>
<td>95</td>
</tr>
<tr>
<td>L-Tryptophan 3 µg</td>
<td>23</td>
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<tr>
<td>L-Tryptophan 8 µg</td>
<td>102</td>
</tr>
<tr>
<td>L-Tryptophan 10 µg</td>
<td>46</td>
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<tr>
<td>L-Tryptophan 20 µg</td>
<td>78</td>
</tr>
<tr>
<td>L-Kynurenine sulfate 10 µg</td>
<td>0</td>
</tr>
<tr>
<td>L-Kynurenine sulfate 50 µg</td>
<td>0</td>
</tr>
<tr>
<td>L-Kynurenine sulfate 100 µg</td>
<td>0</td>
</tr>
<tr>
<td>L-Kynurenine sulfate 400 µg</td>
<td>0</td>
</tr>
<tr>
<td>Anthranilic acid 3 µg + L-Kynurenine sulfate 100 µg</td>
<td>72</td>
</tr>
<tr>
<td>Anthranilic acid 5 µg + L-Kynurenine sulfate 100 µg</td>
<td>37</td>
</tr>
<tr>
<td>L-Tryptophan 3 µg + L-Kynurenine sulfate 100 µg</td>
<td>65</td>
</tr>
<tr>
<td>L-Tryptophan 5 µg + L-Kynurenine sulfate 100 µg</td>
<td>25</td>
</tr>
</tbody>
</table>
from absolute ethanol; 1.7 mg of niacin were obtained; 1.2 mg (determined by assaying the niacin eluted from the planchet) of niacin were counted. If the carbon of niacin was derived solely from the carbon of the indole ring of tryptophan, the niacin counted should have given 383 cpm above background (corrected for the niacin present in the labeled tryptophan preparation). One-sixth of this value would be expected if one carbon of niacin was derived from the indole ring of tryptophan. The isolated niacin did not give any counts above background.

Growth of strain T-2 on labeled indole. The experiment just described was repeated using labeled indole as growth supplement instead of tryptophan. There were two purposes in doing this: first, to confirm with indole the results obtained with the tryptophan preparation (under these conditions tryptophan would be formed endogenously) and second, to examine in vivo the conversion of indole to tryptophan. Labeled indole could be substituted for tryptophan since the strain employed, T-2, responds to either compound.

The experiment performed with labeled indole was essentially the same as the one performed with labeled tryptophan except that labeled indole (2 μg/ml) was used as growth supplement and 400 ml of culture medium were employed. After growth on the medium, the cells were removed by centrifugation and divided into two portions. Half of the cells were utilized for the isolation of tryptophan. These cells were washed once with saline and resuspended in a few ml of water. The cell suspension was poured into cold acetone, and the precipitate which formed was collected by centrifugation. The precipitate was extracted with hot 5 per cent trichloroacetic acid and washed successively with ethanol, ethanol-ether, and finally ether. Fifty-two mg of dry material were obtained. This material was hydrolyzed with 1 N NaOH (1 per cent casein hydrolysate was added, as suggested by Steers and Sevag, 1940) in a sealed glass tube which had been flushed with nitrogen before sealing. After autoclaving for 7 hours the tube was cooled and the contents removed, diluted with a few ml of water, and adjusted to pH 6.0. A small amount of insoluble material was removed by centrifugation. The supernatant solution was then continuously extracted with butanol for 3 hours. The butanol extract was concentrated in vacuo, applied to a sheet of Whatman no. 1 filter paper, and chromatographed in the formic acid solvent previously employed. The tryptophan zone on the chromatogram was located using a pour plate of minimal medium seeded with strain T-1. The tryptophan band of the chromatogram was cut out and eluted with water. The eluate was concentrated in vacuo and the tryptophan content determined microbiologically with strain T-1. This strain responds to the L-isomer of tryptophan only. The eluate gave 5.8 × 10^4 cpm/mg tryptophan. Since the tryptophan isolated was undoubtedly racemic, the corrected value is 2.8 × 10^4 cpm/mg DL-tryptophan. If the indole ring of tryptophan were derived solely from the C^14-indole supplement, 282 × 10^4 cpm/mg would be expected.

Niacin was isolated from the combined acid hydrolysates of half the cells and the concentrated culture filtrate by the procedure described previously except that the niacin eluted from the second chromatogram was counted without further purification and without the addition of carrier. This fraction gave 16.1 cpm above background (the eluate from the planchet contained 10 μg of niacin). If the niacin had been derived solely from the carbon of indole, 350 cpm above background would have been expected.

Since the niacin fraction had some radioactivity, this experiment was repeated to determine whether the radioactivity detected was associated with niacin or with an impurity. Essentially the same procedure was employed as in the previous indole experiment except that 200 ml of medium were used and all the cells were hydrolysed. The niacin eluted from the second chromatogram was not counted, however, but was chromatographed as a single spot in a third solvent system (25 ml butyl alcohol, 50 ml propyl alcohol, and 25 ml of water). The location of the niacin spot on the developed chromatogram was determined as before, and the spot was cut into two sections as illustrated in figure 1. Each section was eluted separately and counted. The top section gave 6.5 cpm above background (1 μg niacin), and the bottom section gave 4.5 cpm above background (7 μg niacin). Thus, the radioactivity does not appear to be associated with niacin but with a slightly faster moving component.

Growth of strain 168 on labeled tryptophan. Tryptophan auxotroph 168 was grown in 750 ml
of minimal medium (0.5 per cent glucose as carbon source) supplemented with 40 ml of the labeled tryptophan preparation. Niacin was isolated from the combined hydrolysates of the cells and the culture filtrate (318 μg niacin), using the same procedure employed in the labeled tryptophan experiment with strain T-2. The final niacin solution, which contained 50 μg of niacin, was diluted 100-fold. The niacin was sublimed and then crystallized from ethanol. The yield was 2.8 mg; 1.2 mg were counted and did not give any counts above background. Three hundred and eighty nine cpm above background (corrected for the niacin content of the tryptophan preparation) would be expected if the carbon of niacin were derived solely from the carbon of the indole ring of tryptophan.

Growth of strain 168 on labeled indole. An experiment with labeled indole as growth supplement was also performed with strain 168. This experiment was identical to the indole experiment performed with strain T-2 except that tryptophan was not isolated from the cells. The niacin counted (20.1 μg) gave 2.8 cpm above background. Seven hundred and five cpm above background would be expected if the niacin were derived solely from the labeled indole.

**DISCUSSION**

Our interest in possible alternate pathways of niacin synthesis was initially aroused by the finding that none of the eleven niacin auxotrophs of *E. coli* and *B. subtilis* which were examined would respond to compounds which were effective niacin substitutes for neurospora mutants. It was appreciated, of course, that all the mutants might be blocked in the last step or steps in niacin synthesis, in which case the compounds tested would not be expected to replace niacin. However, an equally likely possibility was that in these organisms niacin synthesis did not proceed from tryptophan and did not involve kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid as intermediary compounds.

To obtain direct evidence bearing on this problem isotope experiments were carried out with tryptophan auxotrophs of *E. coli* and *B. subtilis*. Since the strains employed require exogenous indole or tryptophan for growth, the niacin formed during growth, if it were derived from tryptophan, could only have come from the exogenous indole or tryptophan. Thus, by labeling the indole or tryptophan supplied, it was possible to determine whether or not niacin was formed from these compounds. Uniformly labeled indole and tryptophan uniformly labeled in the indole ring were used so that niacin synthesis by any of the three possible tryptophan pathways indicated in figure 2 would be detected.

The results of the isotope experiments show conclusively that neither indole nor tryptophan serves as a precursor of niacin in the *E. coli* and *B. subtilis* strains examined. Thus, it is possible to eliminate the three pathways of niacin synthesis illustrated above.

The results obtained would seem to contradict the findings of Marnay. Marnay (1951) demonstrated that in *E. coli* niacin, tryptophan, kynurenine, or 3-hydroxyanthranilic acid would reverse growth inhibition caused by indole-3-acrylic acid. It was concluded from these observations that the pathway of niacin synthesis in *E. coli* is the same as in neurospora. The inhibitory effect of indole-3-acrylic acid was also reversed by phenylalanine, however, which leaves some doubt as to the reaction inhibited by indole-3-acrylic acid. At the present time it is not possible to reconcile Marnay's results and the findings reported in this paper.

Ornithine has been implicated as a precursor of niacin in *E. coli* on the basis of the observation by Ellinger and Abdel Kader (1949b) that this amino acid stimulates niacin production. Other workers have also suggested a role for ornithine as a precursor of niacin (see Rosenberg, 1942). In our experiments ornithine did not sup-
Figure 2. Possible pathways of niacin formation from indole or tryptophan. ● indicates carbon atoms which would be expected to be labeled.

port the growth of any of the niacin auxotrophs of E. coli or B. subtilis which were examined. Furthermore, ornithine does not stimulate niacin production in either of the wild type strains studied. It can be concluded, therefore, that tryptophan does not serve as a precursor of niacin in the K-12 strain of E. coli or the Marburg strain of B. subtilis. Nor does there seem to be any evidence suggesting that ornithine is involved in niacin synthesis in these strains.

Both E. coli and B. subtilis appear to lack kynureninase activity since anthranilic acid responding tryptophan auxotrophs do not respond to kynurenine. This conclusion is supported by the additional finding that the wild type strains of E. coli and B. subtilis do not produce detectable amounts of anthranilic acid when grown in the presence of high concentrations of tryptophan or kynurenine. However, some tryptophan auxotrophs of both organisms (those unable to use anthranilic acid for growth) do form and accumulate anthranilic acid. In view of the absence of kynureninase, this anthranilic acid could not be produced via the tryptophan cycle (Haskins and Mitchell, 1949). Thus, in these organisms anthranilic acid is not a by-product of tryptophan breakdown but is either a "true" intermediate or is readily converted to one of the compounds on the biosynthetic pathway of tryptophan formation.

There are reports of the formation of anthranilic acid from tryptophan or kynurenine by other strains of B. subtilis (Sasaki, 1923; Kotsake and Otani, 1933).

In one experiment the relationship between indole and tryptophan was examined by isolating tryptophan from the protein of a tryptophan auxotroph of E. coli which was grown on labeled indole. The isotope content of the tryptophan isolated indicates that tryptophan is synthesized from indole in E. coli, a finding consistent with the following observations: (1) indole supports the growth of several tryptophan auxotrophs, (2) indole is accumulated by tryptophan auxotrophs of E. coli which cannot use indole for growth, and (3) tryptophan synthetase activity can be demonstrated in extracts of the wild type strain while similar extracts from tryptophan auxotrophs which cannot use indole for growth lack tryptophan synthetase activity. The results obtained also indicate that under the conditions of the experiment there was no "leakage" (Bonner et al., 1952) past the block in the auxotroph employed.

In conclusion it is interesting to note that although both E. coli and Neurospora crassa appear to synthesize tryptophan by the same pathway, tryptophan is used as a precursor of niacin by neurospora but not by E. coli.

ACKNOWLEDGMENTS

The author is indebted to Mrs. Dorothy de la Haba and Mrs. Miriam Bonner for their technical assistance and to Dr. David M. Bonner for
his interest in this investigation. The author also wishes to express thanks to Mr. A. Light for helping with some of the isotope determinations.

SUMMARY

A group of compounds, including several of the known intermediates in niacin synthesis from tryptophan, was found incapable of supporting the growth of three niacin auxotrophs of Escherichia coli and eight niacin auxotrophs of Bacillus subtilis.

Kynureninase, an essential enzyme in niacin synthesis from tryptophan, appears to be absent from the strains examined.

Isotope experiments performed with uniformly labeled indole and tryptophan uniformly labeled in the indole ring showed that neither indole nor tryptophan serves as a precursor of niacin in E. coli and B. subtilis.

Uniformly labeled indole was converted to tryptophan, without dilution, by a tryptophan auxotroph of E. coli.

From the results obtained it can be concluded that neither E. coli nor B. subtilis synthesizes niacin from tryptophan.

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