A QUANTITATIVE STUDY OF THE RELATIONSHIP BETWEEN TRYPTOPHAN AND NIACIN IN NEUROSPORA

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Numerous studies elucidating the probable pathway of tryptophan synthesis (1, 2) and of niacin synthesis (3-9) in Neurospora have been reported. That tryptophan may serve as a precursor of niacin was first suggested by the results of nutritional studies with mammals (10, 11). This relationship has been confirmed for the rat by tracer experiments (12). A tryptophan-niacin relationship has also been suggested for Neurospora (3), based on the behavior of a mutant strain which utilizes either tryptophan or niacin for growth. Although the existence of mutants requiring either tryptophan or niacin for growth suggests a metabolic relationship between these two compounds, mutants of the type mentioned would not be expected if niacin synthesis in Neurospora proceeds solely by way of tryptophan. Mutant strains should be either tryptophan-requiring or niacin-requiring and should not respond to both compounds. It is difficult, therefore, to account for the unorthodox behavior of strains capable of using either tryptophan or niacin for growth, unless the relationship between tryptophan and niacin is more complex than has been postulated, or our understanding of the genetic control of biochemical reactions is inadequate. It was for the purpose of critically examining these points that the studies reported here were performed. It was felt that quantitative isotope data might indicate whether or not niacin synthesis in Neurospora proceeds solely by way of tryptophan and might also offer an explanation for the behavior of mutant strains capable of using either tryptophan or niacin for growth.

A preliminary experiment in this direction was reported (13) in which mutant strain 39401, a strain that responds to either tryptophan or niacin, was grown in the presence of N15-indole. The niacin formed in the mycelium during growth was isolated and it was found that 25 per cent of the niacin synthesized was derived from the indole fed. This experiment demonstrated that niacin is synthesized at least in part from indole in Neurospora, presumably via tryptophan. However, from the limited data obtained it was not possible to account for the niacin which was not de-
rived from the labeled indole; i.e., the major portion of the niacin formed. To account for this portion, more critical isotope experiments have been performed.

EXPERIMENTAL

Mutant Strains—Double mutant strains were used in most of this work. These strains were formed by appropriate genetic methods, and the identity of each double mutant was verified by outcrossing with the standard strain. The mutant strains used in this study and the compounds which support their growth are listed below.

Strain 3416-7655, niacin + tyrosine, phenylalanine, p-aminobenzoic acid and anthranilic acid, indole, or tryptophan
Strain 3416-75001, niacin + anthranilic acid, indole, or tryptophan
Strain 3416-10575, niacin + indole or tryptophan
Strain 3416-C-83, niacin + tryptophan
Strain 39401, indole, tryptophan, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, quinolinic acid, or niacin

The block of strain 3416 was introduced in the above mutants for technical purposes. This block results in the accumulation of quinolinic acid, a product known to be derived from 3-hydroxyanthranilic acid, as is niacin (6, 7), and therefore equivalent to niacin for the purpose of determining the source of the niacin nitrogen. Quinolinic acid was preferred as an end-product because of its heavier yield and greater ease of isolation.

Culture Conditions—In each experiment, 18 liters of sterile minimal medium (supplemented as required for each strain) were inoculated with a conidial suspension of the desired mutant. Since the 3416 block of each double mutant results in an absolute growth requirement for niacin, an excess of niacinamide was included in the medium in each case except that of strain 39401. For strain 39401 it was supplied in suboptimal amount, since it was the only growth supplement required by that mutant. Anthranilic acid and tryptophan, when used, were added to the medium before autoclaving; indole was dissolved in 50 per cent ethanol and added to the hot sterilized medium. The medium for the growth of strain 3416-7655 was further supplemented with optimal amounts of L-tyrosine, DL-phenylalanine, and p-aminobenzoic acid. The medium for the growth of strain 3416-C-83 was supplemented with DL-serine, a compound which stimulates growth of this mutant.

The cultures were grown with forced aeration in a constant temperature room maintained at 30°. At the end of the culture period a sample of the filtrate, containing mycelium and conidia, was removed aseptically.

1 Strain C-83 was kindly supplied by Dr. H. K. Mitchell of the California Institute of Technology.
for reversion tests. The remaining mycelium was then removed by filtration through cheese-cloth, squeezed dry, and kept frozen until it was used for the isolation of tryptophan. The sample taken for reversion tests was allowed to form conidia on complete medium and the conidia were tested for independence of the nutritional supplements required by the original strain. Inoculated flasks of minimal medium and of medium supplemented with the required substances, both separately and in combination, were observed for appearance of growth. In no case was there evidence of a strain having reverted at any mutant locus.

Assay Methods—When quinolinic acid was to be isolated, the filtrates were assayed for this compound by the method of Henderson and Hirsch (14). This assay was also used for the determination of quinolinic acid in the final concentrates and at intermediate stages in the isolation. In several runs the direct microbiological method for quinolinic acid of Jakoby and Bonner (15) was used.

N\textsuperscript{15}-Labeled Substrates—The anthranilic acid used in this investigation was prepared from N\textsuperscript{15}-labeled potassium phthalimide by a modification of the method of Hoogewerff and van Dorp (16). The N\textsuperscript{15}-labeled indole was kindly supplied by Dr. R. Schayer and Dr. D. Shemin of Columbia University. The N\textsuperscript{13} ring-labeled tryptophan was isolated according to the procedure given in this paper from mycelium of strain 3416-75001 grown on N\textsuperscript{15}-labeled anthranilic acid.

Isolation of m-Tryptophan—The moist mycelium, after grinding in a Waring blender, was hydrolyzed in 6 N Ba(OH)\textsubscript{2} under nitrogen at 120° for a period of 8 hours. After cooling, the semisolid mass was crushed and filtered with suction and the solid residue washed with cold water. Most of the remaining barium was then removed by precipitation with CO\textsubscript{2}. The isolation was completed according to the procedure of Cox and King (17).

In some cases the isolated tryptophan was acetylated; the procedure of Derg, Rose, and Marvel (18) was followed.

Isolation of Quinolinic Acid—A modification of the method of Henderson and Hirsch (14) was used. The culture medium (about 17 liters), after removal of the mycelium, was acidified to pH 1 to 1.5 with HCl and passed through a column packed with a mixture composed of approximately 200 gm. each of norit and Hyflo Super-Cel. The absorbent was then stirred with four 500 ml. portions of 0.1 N ammonium hydroxide, each treatment lasting 1 hour. The third and fourth eluates, containing the bulk of the quinolinic acid, were combined and concentrated in vacuo to approximately 30 ml. After filtration, the concentrate was acidified to pH 4.0 and extracted with ether for 24 hours to remove interfering substances. The remaining aqueous solution was further concentrated to 10 ml. and the
pH lowered to 1. A second 24 hour ether extraction removed the quinolinic acid. This extract was submitted to large scale paper chromatography\(^2\) (19) with n-butanol, n-propanol, and water (1:2:1 volume per volume) as developing solvent. The quinolinic acid band, located by its quenching of fluorescence under ultraviolet light and by its characteristic color reaction with ferrous ammonium sulfate, was eluted with 0.1 N ammonium hydroxide. This eluate, after concentration, was rechromatographed with the same solvent, made 0.01 M with respect to ammonia immediately before use. The quinolinic acid eluate, obtained as before, was concentrated to a small volume and crystalline quinolinic acid was obtained upon addition of two-thirds volume of glacial acetic acid and cooling.

Isolation of Anthranilic Acid—As a by-product from the isolation of quinolinic acid from the filtrate of strain 3416-10575 grown on N\(^{15}\)-containing indole, an ether extract containing anthranilic acid was obtained. This was chromatographed on paper with n-butanol, n-propanol, and water (1:2:1 volume per volume), made 0.005 N with respect to ammonia, as developing solvent. The anthranilic acid band was located by its characteristic \(R_F\), its blue fluorescence under ultraviolet light, and its yellow color reaction with \(p\)-dimethylaminobenzaldehyde. This band was eluted with methanol and, after evaporation of the solvent, the crystalline residue was sublimed \textit{in vacuo} and recrystallized twice from ligroin. A yield of 57 mg. of anthranilic acid, melting at 141-143\(^\circ\), was obtained.

Results

The results of the N\(^{15}\) analyses on the compounds fed and the isolated products are shown in Table I. It is seen that in the experiment employing double mutant 3416-C-83 there was essentially no dilution of the tryptophan nitrogen or of the quinolinic acid nitrogen. In the experiments employing double mutants 3416-7655, 3416-75001, and 3416-10575, dilution of both tryptophan nitrogen and quinolinic acid nitrogen was

\(^2\) A modification of the method of Mueller (20) was also used. In this procedure the sample solution was evaporated on a strip of Whatman No. 1 filter paper \(\frac{1}{4}\) inch by 15 inches. In this process the paper was laid on a silicone-coated glass plate and the sample solution was applied rapidly from a pipette. Drying was hastened with the aid of an infra-red lamp and a fan. The sample strip was then sandwiched between the lower edge of a single sheet of heavy filter paper (Schleicher and Schüll lot No. 470A) and a wick of Whatman No. 1 filter paper 2\(\frac{1}{2}\) inches wide, which served to reduce the rate of flow of the developing solvent. The three pieces of paper were held together temporarily by staples, while a double seam of white cotton thread was sewn with a sewing machine. The staples were then removed before development of the chromatogram. This method of large scale paper chromatography has certain advantages in that the sample is quite evenly spread throughout the paper band and that several sample strips can be prepared at once when it is necessary to use more than one chromatogram for a single preparation.
observed. However, in each case the nitrogen of both compounds was
diluted practically to the same extent. The anthranilic acid isolated from

### Table I

#### Results of N$^{15}$ Analyses

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Substrate</th>
<th>Amount per 15 liters</th>
<th>N$^{15}$ content</th>
<th>Duration of culture period</th>
<th>Compound</th>
<th>Dilution factor during isolation</th>
<th>N$^{15}$ content Calculated (a)</th>
<th>N$^{15}$ content Found (b)</th>
<th>Per cent of theoretical (b/a X 100)</th>
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</thead>
<tbody>
<tr>
<td>3416-C-83</td>
<td>DL-Tryptophan</td>
<td>1433</td>
<td>0.456</td>
<td>4</td>
<td>N-Acetyltryptophan</td>
<td>0.417</td>
<td>0.190</td>
<td>0.188</td>
<td>98.9</td>
</tr>
<tr>
<td></td>
<td>DL-Tryptophan</td>
<td>1433</td>
<td>0.456</td>
<td>4</td>
<td>Quinolinic acid</td>
<td>0.135</td>
<td>0.123</td>
<td>0.116</td>
<td>94.3</td>
</tr>
<tr>
<td>3416-10575</td>
<td>Indole</td>
<td>200</td>
<td>4.08</td>
<td>7</td>
<td>N-Acetyltryptophan</td>
<td>2.04</td>
<td>1.60</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indole</td>
<td>200</td>
<td>4.08</td>
<td>7</td>
<td>Quinolinic acid</td>
<td>0.0090</td>
<td>0.0367</td>
<td>0.0276</td>
<td>75</td>
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<tr>
<td>3416-75001</td>
<td>Anthranilic acid</td>
<td>250</td>
<td>34.7</td>
<td>4</td>
<td>Anthranilic acid</td>
<td>3.2†</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anthranilic acid</td>
<td>250</td>
<td>34.7</td>
<td>4</td>
<td>Tryptophan</td>
<td>17.35</td>
<td>13.3</td>
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</tr>
<tr>
<td>3410-7655</td>
<td>Anthranilic acid</td>
<td>300</td>
<td>3.47</td>
<td>7</td>
<td>Quinolinic acid</td>
<td>0.100</td>
<td>3.47</td>
<td>3.59</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Anthranilic acid</td>
<td>300</td>
<td>3.47</td>
<td>7</td>
<td>N-Acetyltryptophan</td>
<td>1.74</td>
<td>0.650</td>
<td>37</td>
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</tr>
<tr>
<td>39401</td>
<td>Anthranilic acid</td>
<td>300</td>
<td>3.47</td>
<td>7</td>
<td>Quinolinic acid</td>
<td>0.340</td>
<td>1.18</td>
<td>0.473</td>
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<tr>
<td></td>
<td>Anthranilic acid</td>
<td>300</td>
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<td>7</td>
<td>Tryptophan</td>
<td>1.74</td>
<td>1.165</td>
<td>67</td>
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</tbody>
</table>

* The N$^{15}$ content of the total tryptophan nitrogen was taken as one-half that of the ring nitrogen, since any liberated N$^{15}$ could constitute only a negligible proportion of the general nitrogen pool from which the amino nitrogen of tryptophan is presumably derived.

† Based on the isotope content of the isolated tryptophan.

the culture filtrate of strain 3416-10575 did not contain any excess N$^{15}$, while the tryptophan isolated from the mycelium formed in the same experiment contained 3.2 atom per cent excess N$^{15}$ in the ring nitrogen. In
the experiment with strain 39401 grown in the presence of N\textsuperscript{15}-containing anthranilic acid, a large proportion of the tryptophan nitrogen was derived from the nitrogen of the added anthranilic acid.

**DISCUSSION**

As was mentioned in the introduction to this paper, the initial suggestion that a metabolic relationship exists between tryptophan and niacin in *Neurospora* (3) was based on the behavior of a mutant strain whose niacin requirement is satisfied by tryptophan. Supporting evidence for a relationship between tryptophan and niacin came from the observation that a known product of tryptophan metabolism, kynurenine, would replace niacin for a *Neurospora* mutant (3). The demonstration that large quantities of a derivative of kynurenine, α-N-acetylkynurenine, are abnormally accumulated by a second niacinless mutant (8) also serves to link tryptophan metabolism with niacin synthesis.

Although a relationship between tryptophan and niacin was indicated by the evidence mentioned, the question as to whether or not niacin formation from tryptophan constitutes the major pathway of niacin synthesis in *Neurospora* remained unanswered. In the experiments reported in this paper a combination of mutant strains of *Neurospora* and labeled substrates provided the experimental materials with which to test this point critically. It was argued that the nitrogen of the niacin synthesized by a tryptophanless mutant should reflect the same isotope content as the ring nitrogen of the labeled tryptophan supplied for growth, provided niacin was synthesized solely by way of tryptophan. If niacin could be formed via some other pathway not involving the ring nitrogen of tryptophan, this would result in the formation of niacin with a lower N\textsuperscript{15} content than the ring nitrogen of the tryptophan fed. Actually, in these experiments double mutants were employed and quinolinic acid was isolated instead of niacin, for reasons mentioned earlier in this paper. Preliminary isotope experiments\textsuperscript{3} indicated that the nitrogen of quinolinic acid and the nitrogen of niacin were equivalent, a conclusion also arrived at from other studies (6, 21) which showed that both compounds are derived from the same precursor, 3-hydroxyanthranilic acid.

In the experiment performed with double mutant 3416-C-83, N\textsuperscript{15} ring-labeled tryptophan was fed and the results obtained (see Table I) show that the quinolinic acid synthesized by this mutant was derived solely from the labeled tryptophan supplied. Tryptophan was also isolated at the end of the culture period and no dilution of its nitrogen was observed.

\textsuperscript{3} Additional N\textsuperscript{15} tracer experiments performed in this laboratory have shown that niacin and quinolinic acid are both derived quantitatively from the same added precursor, 3-hydroxyanthranilic acid.
This result shows that, under the culture conditions employed, strain 3416-C-83 is incapable of tryptophan synthesis. Since in this case a block in a single step, the conversion of indole to tryptophan, results in the inability of the double mutant concerned to synthesize quinolinic acid from sources other than the labeled tryptophan supplied, quinolinic acid synthesis must involve tryptophan as a sole precursor. This experiment also rules out the possibility of a direct oxidation of anthranilic acid to 3-hydroxyanthranilic acid (as appears to be the case in Aspergillus (22)).

A large amount of anthranilic acid, in addition to a small amount of indole, is accumulated by strain 3416-C-83. In the experiment just discussed, this anthranilic acid would be principally unlabeled (a small portion could be formed from the labeled tryptophan via the tryptophan cycle (9)), and, if a direct oxidation to 3-hydroxyanthranilic acid were possible in Neurospora, should lead to a dilution of the nitrogen of quinolinic acid. This was not observed.

Similar quantitative isotope studies were performed with three additional double mutants, 3416-10575, 3416-75001, and 3416-7655. Employing either N15-labeled anthranilic acid or indole as substrate, in each experiment (see Table I) good agreement was obtained between the isotope content of the tryptophan and quinolinic acid synthesized. Unexpectedly, it was found that the isolated products were diluted in the experiments with these three double mutants. However, approximately the same dilution was obtained in the tryptophan and quinolinic acid synthesized in each experiment. This fact suggests that tryptophan is diluted first and this dilution then shows up in quinolinic acid. This would account for the slightly high quinolinic acid value obtained in the 4 day growth experiment with strain 3416-75001.

Thus the evidence obtained indicates that quinolinic acid synthesis, and therefore niacin synthesis, proceeds solely by way of tryptophan. If alternate pathways of niacin synthesis do exist in Neurospora, they are of minor importance, at least under the experimental conditions employed in these investigations.

The evidence obtained also confirms the conversion of anthranilic acid and indole to tryptophan by Neurospora (1, 2). Haskins and Mitchell (9) have questioned the participation of anthranilic acid as a direct intermediate in tryptophan synthesis and have proposed a scheme (the tryptophan cycle) in which anthranilic acid is formed in two ways; from tryptophan by way of kynurenine and from a hypothetical indole precursor with which it is in equilibrium. Although it is conceivable that anthranilic acid is not a direct intermediate in tryptophan synthesis, Haskins and Mitchell have not presented any experimental evidence on which to base the conclusion that indole is formed without anthranilic acid participating.
as an intermediate. Such evidence would be required if anthranilic acid is to be excluded from the scheme of tryptophan synthesis.

The significance of the tryptophan cycle was evaluated in the experiment performed with double mutant 3416-10575, a strain which accumulates anthranilic acid. This strain was grown on N$^{15}$-labeled indole and anthranilic acid was isolated from the culture filtrate at the end of the culture period. The isotope analysis (see Table I) revealed that there was no excess N$^{15}$ in the isolated anthranilic acid. Tryptophan, isolated from the mycelium obtained in the same experiment, contained 3.2 atom per cent excess N$^{15}$ in the ring nitrogen. From these values it can be calculated that no more than one-thousandth of the anthranilic acid yield in this experiment could have come from tryptophan; otherwise it would have been detected. These results suggest that, when tryptophan is produced gradually from its normal precursors rather than added directly to the culture medium (as in the experiments of Haskins and Mitchell), the operation of the tryptophan cycle may be greatly reduced or entirely eliminated.

Since niacin is synthesized solely by way of tryptophan in Neurospora, we are confronted with the problem of accounting for the unorthodox behavior of the mutant strains which are capable of using either tryptophan or niacin for growth. All such strains appear to be blocked at steps in tryptophan synthesis, and, like other tryptophanless mutants, both their tryptophan and niacin requirements are satisfied by an exogenous supply of tryptophan. However, in distinction to the tryptophanless mutants which do not respond to niacin, these mutants do. Niacin cannot be seriously considered as a precursor of tryptophan and thus account for the ability of certain strains to use either compound for growth, because the amount of niacin required to support growth is far too small. Therefore it must be concluded that such strains are capable of tryptophan synthesis from the constituents of minimal medium under growing conditions. This conclusion was further examined by growing strain 30401, a mutant that does not respond to anthranilic acid, on niacinamide in the presence of N$^{15}$-labeled anthranilic acid. Tryptophan was isolated from the resulting mycelium and analyzed for its N$^{15}$ content (see Table I). It was found that 67 per cent of the tryptophan synthesized by the mutant was derived from the labeled anthranilic acid. Thus this mutant can use anthranilic acid efficiently as a precursor of tryptophan when it is grown on niacin. The fact that only 67 per cent of the tryptophan was derived from the labeled anthranilic acid can be explained on the assumption that the remainder was derived from unlabeled anthranilic acid synthesized by the mutant. Thus this mutant, under growing conditions, converts anthranilic acid to tryptophan, and would appear to be capable of form-
ing tryptophan by the normal sequence of reactions. This mutant, then, can synthesize an essential compound from a precursor which growth data suggest that it is incapable of using. This ability of a mutant strain to form those compounds which it specifically requires for growth has been termed "leakage" (23). Leakage also occurs in three of the four double mutants which were employed in this study (see Table I).

Following the observation that strain 39401 is capable of converting anthranilic acid to tryptophan when grown on niacin, this strain was tested on a wide range of anthranilic acid concentrations. Although it had been known for some time that this strain does not respond to "normal" concentrations of anthranilic acid, it was found that high concentrations of the order of 20 times normal would support growth on an otherwise unsupplemented medium. It has also been found that the ability of strain 39401 to use anthranilic acid for growth is appreciably affected by several modifier genes. In fact the 39401 locus within certain genetic constitutions permits substantial growth on anthranilic acid.

Thus all the evidence supports the view that niacin is synthesized solely by way of tryptophan in Neurospora. That strains are known whose behavior would appear to contradict this relationship does not indicate that our understanding of the biochemistry of this problem is at fault. Rather it suggests that genetic changes have rendered such strains incapable of synthesizing sufficient tryptophan to supply both the tryptophan and niacin required for growth, and that it is our understanding of the genetic control of biochemical reactions which is inadequate.

SUMMARY

A quantitative investigation of the tryptophan-niacin relationship in Neurospora was carried out. Four mutant strains requiring tryptophan for growth, each genetically blocked in different steps in tryptophan formation, were cultured on tryptophan, indole, or anthranilic acid containing excess N^{15}. In each case the tryptophan of the formed mycelium was isolated, and its N^{15} content determined. The quinolinic acid accumulated in the culture medium as the result of the incorporation of a second genetic block was also isolated in each case and analyzed for N^{15}. From these investigations it may be concluded (1) that the nitrogen of anthranilic acid and indole is converted to the ring nitrogen of tryptophan and niacin, and (2) that niacin is synthesized solely by way of tryptophan in Neurospora.

Strain 39401, which utilizes either tryptophan or niacin but not anthranilic acid for growth, was cultured on niacin in the presence of N^{15}-anthranilic acid. The ring nitrogen of the tryptophan isolated was found to contain 67 per cent of the concentration of N^{15} present in the added
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anthranilic acid. This permits the conclusion that this strain is capable of independent tryptophan synthesis under growing conditions.

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BIBLIOGRAPHY

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