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Francis A. Haskins
University of Nebraska - Lincoln, fhaskins@neb.rr.com

Herschel Mitchell
The Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

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EVIDENCE FOR A TRYPTOPHANE CYCLE IN NEUROSPORA*

BY FRANCIS A. HASKINS AND HERSCHEL K. MITCHELL

THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY
PASADENA, CALIFORNIA

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The work of Snell[1] on lactic acid bacteria provided the first evidence that anthranilic acid may act as a precursor to tryptophane *in vivo*. Since the strains of bacteria which utilize anthranilic acid in the place of tryptophane also use indole, it was not possible to establish the order in which these two precursors occurred. In 1943 Tatum, Bonner and Beadle[2] found that anthranilic acid was accumulated in the culture medium by a *Neurospora crassa* mutant, strain 10575, which required either tryptophane or indole for growth. It was therefore indicated that anthranilic acid is a precursor to indole in Neurospora.

It has recently been observed in this laboratory that when a medium containing tryptophane is inoculated with wild type or any one of a number of tryptophane-utilizing strains of Neurospora, a blue fluorescence, resembling that of an anthranilic acid solution, is produced. The fluorescence is detectable within a few hours after inoculation, reaches a maximum intensity at 24 to 30 hours and then diminishes until, at 72 hours, the medium displays essentially no fluorescence except in the case of the mutants which are able to use tryptophane but cannot utilize anthranilic acid. Earlier work has already demonstrated that anthranilic acid is formed by the degradation of tryptophane by bacteria[3, 4] and in the rat and other animals.[5] Thus the appearance and subsequent disappearance of fluorescence in Neurospora medium suggested that in Neurospora some of the reactions by which tryptophane is degraded and synthesized may constitute a metabolic cycle. The present experimental evidence supports this conclusion.

An earlier communication[6] from this laboratory postulated 3-hydroxykynurenine as an intermediate in the conversion of tryptophane to nicotinic acid in Neurospora. Through the generosity of Dr. W. Weidel, of the Kaiser Wilhelm Institut für Biochemie, a sample of this compound was made available to the authors and it became possible to establish its relation to the tryptophane cycle in Neurospora. The substance was isolated by Weidel in 1949 from larvae of *Calliphora erythrocephala* as the cn^+ substance (precursor of eye pigments in insects).

Experimental.—Mutant Strains.—The qualitative growth responses of the various Neurospora strains considered in this investigation are listed in table 1. Previous descriptions have been made of strains E5212,[7] C86,[7, 8] 10575,[2] C83[8, 9] and 4540.[10]

Disappearance of Tryptophane from the Culture Medium.—Erlenmeyer

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flasks (125 ml.) containing 20 ml. of minimal medium[11] supplemented with 400 γ of L-tryptophane were inoculated with conidia from Neurospora strain B1312 and were incubated at 25°C. Absorption spectra of the medium before inoculation and at 8-hour intervals thereafter were determined with a Beckman spectrophotometer. These spectra are shown in Figure 1. Dry weights of mold per flask at the different times were as follows: 16 hours, 0.6 mg.; 24 hours, 3.0 mg.; 32 hours, 9.1 mg. The subsequent dry weight attained at 96 hours was 55 mg. On the basis of the spectra at 280 mμ (the absorption maximum for the indole ring of tryptophane), it is obvious that at least 100 γ of the tryptophane are gone when the growth of mold is only 0.6 mg. By the time 3 mg. of growth has been produced, at least half of the tryptophane has disappeared, and the shape of the absorption spectrum suggests that actually much more than half is gone.

In order to eliminate the possibility that the tryptophane was not simply absorbed from the medium by the small amount of mold present in the early stages of growth, the mold was removed from 32- and 96-hour cultures,

TABLE 1

UTILIZATION OF VARIOUS COMPOUNDS BY *Neurospora* MUTANTS

STRAIN	TYROSINE AND CINNAMIC ACID	PHENYL-ALANINE	ANTHRA-NILIC ACID	INDOLE	TRYPTO-PHANE	KYNU-RENINE	3-HY-DROXY-KYNU-RENINE	3-HY-DROXY-ANTHRA-NILIC ACID	NICO-TINIC ACID
E5212	−	+	−	−	−	−	−	−	−
C86	+	+	+	+	+	+	+	+	+
B1312	−	−	+	+	+	+	−	−	−
10575	−	−	−	+	+	−	−	−	−
C83	−	−	−	−	+	−	−	−	−
E5029	−	−	−	−	−	−	+	+	+
4540	−	−	−	−	−	−	−	−	+

hydrolyzed with 1 *N* NaOH, and the tryptophane content was determined by bioassay with *S. lactis R.*[1] A control sample of tryptophane was subjected to the same treatment. The tryptophane content of the 32-hour culture on a dry-weight basis was found to be 0.3% and that of the 96-hour culture 0.2%. After 32 hours the mold therefore contains approximately 27 γ of tryptophane as opposed to a disappearance of at least 200 γ. Thus it is clearly demonstrated that the tryptophane has not been taken up as such by the mold, but has actually been converted to other compounds.

Products of Tryptophane Degradation.—Flasks of medium supplemented with 400 γ of L-tryptophane per 20 ml. were inoculated with conidia from wild-type strain 8a and from mutants B1312, C86 and 10575, respectively. After 32 hours' incubation at 25° the mold was removed from the flasks, the medium was filtered, the pH was adjusted to 2.5 and the medium was extracted four times with an equal volume of ether. The ether was evaporated on a warm water bath. With each strain the absorption spectrum of

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the material in the ether phase was found to be nearly the same as the absorption spectrum of the unextracted medium. Measurements of fluorescence using a Coleman photofluorometer disclosed that approximately 80% of the fluorescence originally present in the unextracted medium went to the ether phase. The residual aqueous phase had an absorption spectrum corresponding to a maximum of 50 γ of tryptophane. Bioassays, using strains of C86 and B1312, showed that from the original 400 γ of tryptophane there remained in the medium after 32 hours' growth the biological equivalent of approximately 250 γ. Of this, approximately 180 γ were extracted with ether. With the unextracted medium, strains 10575 and C83 which

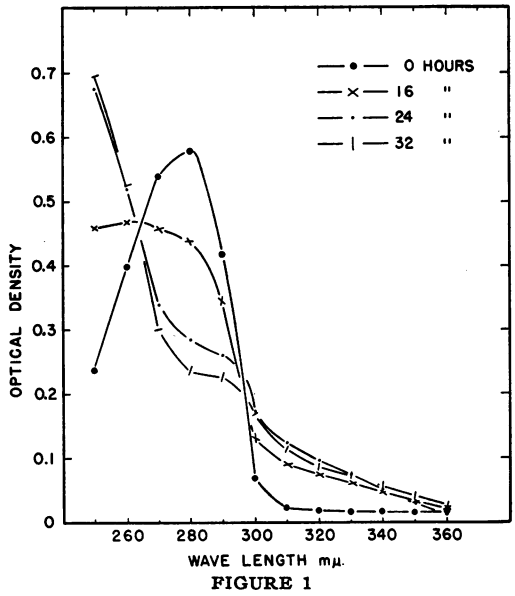

FIGURE 1

Absorption spectra of *Neurospora* medium at various times after inoculation.

do not use anthranilic acid gave growth which was equivalent to that obtained on about 50 γ of tryptophane, but neither of these strains grew on the ether extract. All four of the strains tested gave the same general qualitative and quantitative results as determined by measurements of absorption spectra, fluorescence and biological activity.

Filter paper strip chromatograms were run on the ether extract with water-saturated butanol as the solvent. After development, the chromatograms were examined under ultra-violet light to detect fluorescent areas, and were then cut into sections, the sections were extracted with 0.1 *M* KH_2PO_4 (pH 3.5), and the biological activities of the resulting solutions were determined using B1312 for the assays. It was found that approximately 60% of the activity of the ether phase could be accounted for by the

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activity of a chromatogram band which resembled an anthranilic acid band in its position and fluorescence.

In order to obtain enough material for isolation of some of the degradation products, 8 liters of culture medium containing 8 g. of L-tryptophane was inoculated with a heavy inoculum of conidia from wild-type strain 8a. The culture was incubated with forced aeration in a 35° water bath for 19 hours. The mycelium was then filtered off, the pH of the filtrate was adjusted to 2.5, the filtrate was divided into two portions and each was extracted with seven 500-ml. portions of ether. Following evaporation of the ether, the residual solution was absorbed on 29 sheets of filter paper and these sheets were incorporated into a chromatopile[12] consisting of 600 sheets of 9-cm. Whatman No. 1 filter paper. Water-saturated butanol was used as the developer. After 15 hours, every twentieth sheet was removed from the pile and the location of anthranilic acid was determined by absorption spectra of extracts of the sample sheets. The desired section was then removed from the chromatopile, dried in a vacuum desiccator over $CaCl_2$ and extracted with ether in a Soxhlet. The residue from evaporation of the ether was sublimed at 100° *in vacuo* (20 microns Hg). The sublimate was crystallized from water and further purified by resublimation in a temperature gradient at 20 microns pressure. The material obtained in this way melted at 143–144° (cor.). A known sample of anthranilic acid melted at 144–145° (cor.) and a mixture of the two melted at 144–145° (cor.). The neutralization equivalent of the isolated compound was 137 (calculated for anthranilic acid—137). The absorption spectrum, fluorescence and biological activity were identical with that of anthranilic acid, furnishing further proof that the isolated compound was indeed anthranilic acid.

Chromatographic examination of the products of tryptophane degradation by germinating Neurospora has demonstrated the existence of at least two biologically active substances besides anthranilic acid. In addition bio-assay with *L. arabinosus* has shown the production of 4 γ of nicotinic acid from 400 γ of tryptophane in 32 hours.

A number of compounds other than tryptophane were tested to determine whether they were able to give rise to fluorescent degradation products in the medium. Strain C86 was used as the test strain. It was found that strain C86 produced the typical blue fluorescence in the medium when supplied with phenylalanine, indole or kynurenine. No appreciable fluorescence was produced when the medium was supplemented with trans-cinnamic acid, tyrosine, 3-hydroxykynurenine or nicotinamide. A test on 3-hydroxyanthranilic acid appeared also negative but inasmuch as the compound itself had some blue fluorescence the test was not critical. In the cases where fluorescence was produced it was not permanent but reached a maximum and then disappeared, as did the fluorescence produced when C86 was supplied with tryptophane.

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Discussion.—The evidence reported here establishes the fact that tryptophane disappears rapidly from culture medium which has been inoculated with wild-type Neurospora strain 8a or mutant strains C86, B1312 or 10575. A large part of the tryptophane is gone before appreciable growth of the mold has occurred. It is also established that anthranilic acid is one of the products of tryptophane breakdown in Neurospora.

The accumulated evidence points clearly to the existence in Neurospora of a metabolic cycle involving, among other compounds, tryptophane and anthranilic acid. A schematic representation of such a cycle is shown in

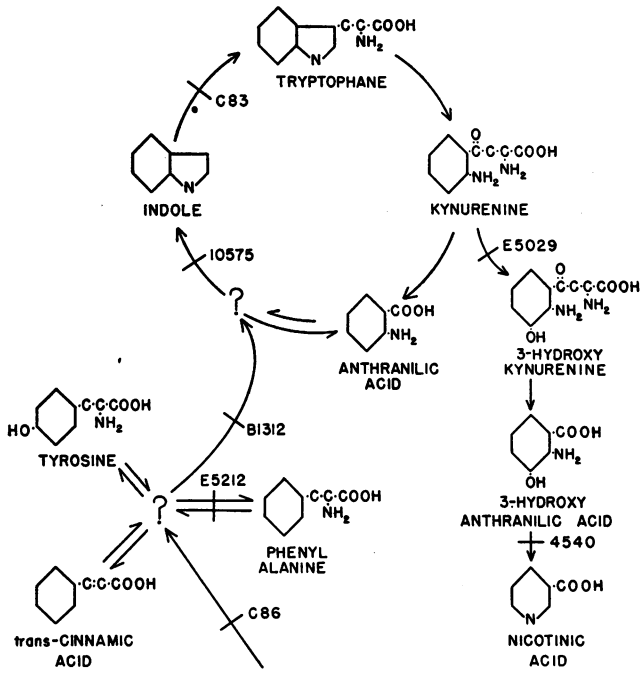

FIGURE 2
Schematic representation of a tryptophane cycle in *Neurospora*.

figure 2. Since strain B1312 is able to utilize anthranilic acid or kynurenine, but not 3-hydroxykynurenine, it is apparent that kynurenine is an intermediate in the formation of anthranilic acid from tryptophane and that 3-hydroxykynurenine is not. In addition the mold will produce blue fluorescence in the medium when supplied with kynurenine, but not when supplied with the hydroxy compound. The reactions by which anthranilic acid is formed from tryptophane must include at least one essentially irreversible step, for strains 10575 and C83 cannot utilize anthranilic acid although they

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are able to make it from tryptophane. This is in agreement with the finding by Kotake[5] that rats can make anthranilic acid from tryptophane, but are evidently unable to use anthranilic acid to satisfy their tryptophane requirement.

As previously reported[13] the carboxyl carbon atom of anthranilic acid is lost as CO_2 during metabolism of this substance by Neurospora. It seems most probable that this occurs in the conversion of anthranilic acid to indole since Heidelberger has traced the corresponding carbon of the indole ring of tryptophane to kynurenine and nicotinic acid in rats.[14, 15, 16] For this reason anthranilic acid has tentatively been excluded as an intermediate between phenylalanine and indole in the diagram in figure 2. The accumulation of anthranilic acid by strain 10575[2] is not sufficient reason for placing anthranilic acid immediately before indole since strain C83 also accumulates this substance. It seems more likely that the accumulation of anthranilic acid is due to a reversible reaction between this compound and an unknown intermediate with anthranilic acid formation being favored as shown in the diagram. The accumulation of anthranilic acid during tryptophane degradation by wild type is in agreement with this conclusion. This problem requires further elucidation before the position of anthranilic acid in the cycle can be established with certainty. The number or nature of all the intermediates in the cycle is not known, but as stated above, there are at least three biologically active substances other than anthranilic acid produced as the result of tryptophane degradation. The task of isolating and identifying these intermediates and side products is in progress.

It is clear from the experimental evidence that at least one-fourth of the tryptophane degraded by the mold is converted to substances lacking biological activity for the tryptophane series of mutants. It is not yet clear, however, whether or not these compounds or the cycle reactions play essential roles in the metabolism of the mold. It is to be noted that the mutants which cannot carry out reactions within the cycle (10575 and C83) grow poorly, as compared to all the other mutants of the series, even with an excess of tryptophane. This fact suggests a special metabolic significance to turnover in the cycle.

Summary.—1. Tryptophane is shown to disappear rapidly from culture medium which is inoculated with any one of the Neurospora strains tested. One of the products of tryptophane breakdown has been isolated and identified as anthranilic acid.

2. Evidence is presented which suggests that in Neurospora tryptophane, anthranilic acid and several other compounds are involved in a metabolic cycle.

3. The evidence demonstrates that 3-hydroxykynurenine is an intermediate in tryptophane metabolism in Neurospora, but indicates that it is not directly involved in the cycle.

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