EVIDENCE FOR THE PARTICIPATION OF KYNURENINE AS A NORMAL INTERMEDIATE IN THE BIOSYNTHESIS OF NIACIN IN NEUROSPORA*

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Investigations on the mechanism of the biological synthesis of niacin have implicated tryptophan and 3-hydroxy-anthranilic acid as niacin precursors in both Neurospora[1, 2] and the rat.[3, 4] 3-Hydroxy-anthranilic acid has been shown to serve as a normal intermediate in niacin synthesis in Neurospora,[5] and a mechanism for the conversion of this compound to niacin has been suggested.[6] It was not until recently, however, that conclusive proof was obtained that tryptophan normally serves as a major precursor of niacin in Neurospora.[7] As yet no clear proof of the identity of the intermediates between tryptophan and 3-hydroxy-anthranilic acid has been obtained. Kynurenine and 3-hydroxy-kynurenine have been proposed as intermediates in the conversion of tryptophan to niacin in Neurospora.[2] Kynurenine has been tested for its ability to support the growth of rats maintained on a niacin deficient diet and found inactive.[8] However, recent evidence presented by Heidelberger[9] and by Kallio[10] suggests that kynurenine may play a role in the conversion of tryptophan to niacin by the rat.

In the Neurospora investigations evidence substantiating the proposal that kynurenine and 3-hydroxy-kynurenine function as natural precursors of niacin has not yet been presented. Although both of these substances have been tested and found highly active in supporting the growth of certain niacinless strains,[1, 11] proof of their actual participation in niacin synthesis is still lacking. This lack of evidence is primarily due to the fact that mutant strains capable of utilizing 3-hydroxy-anthranilic acid and niacin but incapable of using tryptophan have not been studied. Mutants of this class might accumulate one or more of the intermediates between tryptophan and 3-hydroxy-anthranilic acid, and accumulation of either kynurenine or 3-hydroxy-kynurenine would constitute direct proof of the participation of these compounds in the biosynthesis of niacin in Neurospora. A mutant strain has been obtained[5] which can use 3-hydroxy-anthranilic acid, cannot use tryptophan and does accumulate a kynurenine-like compound. The present paper deals with the isolation and identification of this substance and the significance of this accumulation.

Experimental.—The basal medium used throughout is the customary Neurospora minimal.[12] Cultures were grown 72 hours at 25°C. in 125-ml. Erlenmeyer flasks containing 20 or 40 ml. of medium for growth tests. At the end of the period of incubation the mycelial pads were removed,

dried and weighed. Test samples were added to the assay flasks before autoclaving.

Table 1 lists the various Neurospora mutants used in this investigation and the substances known to support their growth.

Strain Y-31881 is biochemically distinct from the other mutant strains listed, since the group of compounds which will support its growth is characteristic of it alone. The growth of this strain is supported by any one of the following compounds: 3-hydroxy-kynurenine,† 3-hydroxy-anthranilic acid, quinolinic acid at high concentrations and niacin. Tryptophan, its precursors or kynurenine cannot replace niacin for this mutant. Genetic tests demonstrate that Y-31881 differs from the parental strain by a single gene mutation. That this strain differs genetically from the other strains listed in table 1 was concluded from the fact that it forms a heterocaryon (as a test for allelism)[13] with these strains, and that from crosses with each of the strains listed in table 1, nutritionally wild-type progeny were recovered.

TABLE 1

GROWTH OF VARIOUS STRAINS OF NEUROSPORA

STRAIN NO.	NO ADDITION	ANTHRANILIC ACID	INDOLE	TRYPTOPHAN	KYNURENINE	α-N-ACETYL KYNURENINE	3-HYDROXY-KYNURENINE	3-HYDROXY ANTHRANILIC ACID	QUINOLINIC ACID	NIACIN
75001	−	+	+	+	+	−	?	−	−	−
10575	−	−	+	+	−	−	−	−	−	−
39401	−	−	+	+	+	+	+	+	±	+
Y-31881	−	−	−	−	−	−	+	+	±	+
4540	−	−	−	−	−	−	−	−	±	+
3416	−	−	−	−	−	−	−	−	−	+
Y-31881-3416	−	−	−	−	−	−	−	−	−	+
Wild type	+	+	+	+	+	+	+	+	+	+

On the basis of these observations it was concluded that this mutant is genetically blocked in the conversion of kynurenine to 3-hydroxy-anthranilic acid. The double mutant Y-31881–3416 permitted a direct check on this interpretation. Strain 3416 is a niacin-requiring mutant which cannot use 3-hydroxyanthranilic acid in place of niacin, and accumulates quinolinic acid freely in its culture medium.[6] If the Y-31881 block interfered with the synthesis of 3-hydroxy-anthranilic acid, which in turn is known to give rise to quinolinic acid, it would be expected that this double mutant should no longer accumulate this acid. The opposite mating types of the two strains concerned were crossed and ascospores were isolated in order from several asci. In one ascus four wild-type spores were obtained, indicating that the other four must be double mutants. Crossing a presumed double mutant with wild type gave both individual mutant types, verifying the genotype of the double mutant. Y-31881–3416 and 3416,

one of the parental types, were grown on a medium supplemented with niacin and the amount of quinolinic acid accumulated determined. It can be seen from table 2 that the double mutant Y-31881–3416 does not accumulate an appreciable amount of quinolinic acid when compared to strain 3416. The Y-31881 block must then prevent an enzymatic reaction which normally leads to the production of a quinolinic acid precursor.

Cross-feeding experiments were performed to test for the accumulation of an active niacin precursor in the culture filtrates of strain Y-31881. Strain 39401 was selected as the assay organism since this strain would be expected to grow in the presence of indole, tryptophan or any of the intermediates between tryptophan and niacin (see table 1). The results of a typical experiment are shown in figure 1. It is clear that these filtrates contain a substance or substances which support the growth of strain 39401 but not strain Y-31881. Since the growth of strain 39401 alone is supported by Y-31881 culture filtrate, it may be concluded that the accumulated substance is not 3-hydroxy-kynurenine, 3-hydroxy-anthranilic acid, quinolinic acid or niacin, since both strains are equally sensitive to these compounds.

To obtain sufficient material for the isolation of the active substance (designated as 31881-I), strain Y-31881 was grown in 5-gallon bottles con-

TABLE 2

QUINOLINIC ACID ACCUMULATION BY VARIOUS MUTANT STRAINS OF NEUROSPORA

STRAIN	SUPPLEMENT	γ-QUINOLINIC ACID PER CC. FILTRATE	γ-QUINOLINIC ACID PER MG. DRY WEIGHT
3416	20 γ-nicotinamide	50.5	8.3
Y-31881-3416	20 γ-nicotinamide	0.49	0.14

taining 18 liters of half-strength minimal medium supplemented with 4 mg. of nicotinamide. Half-strength minimal was used instead of normal strength since it did not decrease the amount of 31881-I accumulated yet reduced the difficulty of isolation. The cultures were incubated at 25°C. under continuous aeration. After 7–10 days of growth the mycelium was removed by filtering through cheesecloth. The filtrates from two bottles were combined and concentrated *in vacuo* to approximately two liters. The concentrate was filtered to remove insoluble material, which was then washed twice with ethanol. The washings were added to the clear concentrate. Two volumes of ethanol were added and the mixture cooled overnight. The precipitate formed was filtered off and washed as before. The clear solution was evaporated almost to dryness, brought up to a volume of 300 cc. with distilled water and acidified to pH 3.5–4.0. It was then continuously extracted with ether for 48 hours. The ether extract was concentrated to ca. 10 cc. and enough ethanol added to bring the final volume to 60 cc. This extract was chromatographed using whole sheets of Whatman No. 1 filter paper cut to 17 × 20 inches. Twelve sheets were

run at the same time and constituted a single batch. Five cc. of the ethanol extract were applied to each sheet by means of a modified kymograph previously described.[14] The sheets were then developed as ascending chromatograms with a butyl alcohol, propyl alcohol, water (1:2:1) solvent made 0.005 *M* with respect to ammonia immediately before use. After 24 hours the sheets were removed and air-dried.

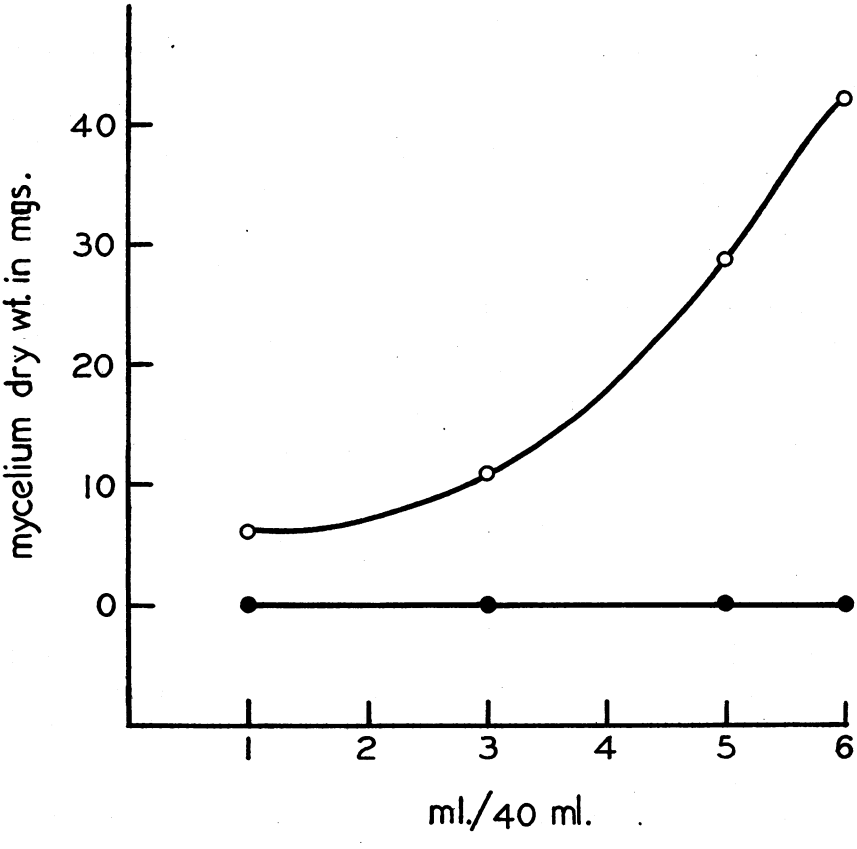


FIGURE 1

Growth of strains 39401 and Y-31881 on culture filtrates of strain Y-31881. ○–○ strain 39401, ●–● strain Y-31881.

The band of active material was next located in the following manner. Sterile modified Neurospora minimal medium was inoculated with a filtered conidial suspension of the test strain and poured into large ($5^1/_2 \times$ 16 inch) plates.[15] The medium used contained inorganic salts, 2% agar, 0.25% sucrose and 1% sorbose, this latter sugar being used to prevent spreading growth of the mycelium.[16] Strains 39401 and Y-31881 were

used as test organisms. Two vertical sections each 1 cm. in width were cut from one sheet of each batch. These strips were sterilized by exposure to a Sterilamp for ca. 15 minutes, after which one strip was placed on a 39401 plate and its duplicate on a Y-31881 plate. Each plate could accommodate 3 strips with sufficient space between them to prevent overlapping of the growth zones. The plates were then maintained at 25°C.

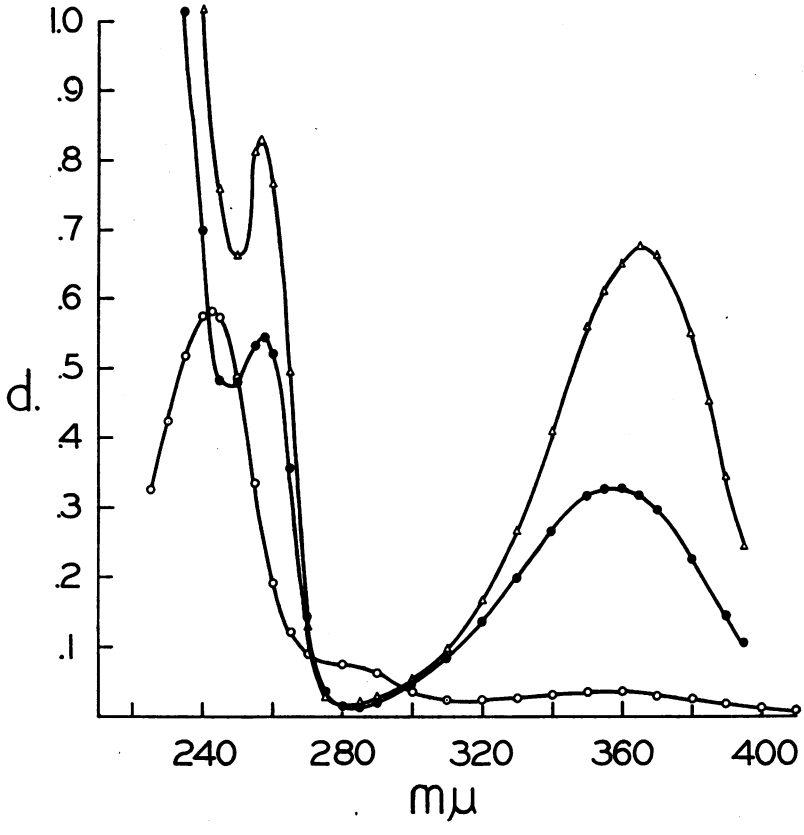


FIGURE 2

Absorption spectra of 31881-I in various solvents. ○–○ 0.1 *N* HCl; ●–● 0.1 *N* NaOH; △–△ abs. ethanol.

for 24 hours. Contaminants were seldom encountered because of the short period of incubation. The areas supporting the growth of 39401 but not Y-31881 were marked and recorded, and the corresponding sections from all sheets of the same batch were cut out and combined. These sections were eluted with dilute ammonia in a Waring blendor. The suspension was filtered to remove the paper pulp, this process repeated

and the eluates combined. The paper pulp was tested for residual activity before being discarded, and it was seldom found necessary to elute a third time. The combined eluates were evaporated to dryness *in vacuo* and the minimum amount of hot ethanol added to effect complete solution. On cooling, light brown needles which supported the growth of strain 39401 appeared. The needles were filtered off and recrystallized from water after norite treatment. This material remained active for growth of strain 39401, but was found to be only about one seven hundredth as active as niacin. After the original isolation procedure was evolved and several mg. of 31881-I were obtained, it was found that the isolation procedure could be considerably shortened. The ether extract was dissolved in hot ethanol, and when the solution had been kept in the refrigerator for a few days, crude crystals of 31881-I appeared. These crystals were filtered off and recrystallized as before.

The product in either case is pale yellow, melts from 190–195°C. (uncorr.) and decomposes at 210°C. with the sublimation of a second substance. The sublimate melts at 237–240°C. (uncorr.) when heated rapidly. The absorption spectra of the isolated material (31881-I) in several solvents are given in figure 2. Of the many substances tested only *o*-aminoacetophenone showed approximately the same absorption spectra in the solvents used, suggesting a possible relationship.

The isolated material is soluble in methanol, ethanol and acetic acid, slightly soluble in water and very slightly soluble in ether. Equivalent weight determinations gave a value of 250. The fact that molecular weight determination by ebulliometry gave values around 250, suggests that the equivalent weight determined is the molecular weight of the substance. Tests for functional groups indicated the presence of an aromatic amino group, a free carboxyl group and the absence of free α-amino, phenolic and alkoxyl groups. When 31881-I was subjected to acid hydrolysis (1 *N* H_2SO_4 at 100°C. for 2 hours), a considerable increase in niacin activity was noticed (Fig. 3). It was also found that the hydrolyzed solution was active for strain 75001 (see table 1) and now gave a positive test for an α-amino group. These facts suggested that the compound formed on hydrolysis was kynurenine. To establish this fact the isolation of kynurenine was attempted. One hundred mg. of 31881-I were hydrolyzed with 1 *N* H_2SO_4, the H_2SO_4 content brought up to 5% (by volume) and enough ethanol added to make an 80% solution. After two days in the refrigerator the colorless crystals were filtered off. These needles gave a strong qualitative test for kynurenine, contained sulfate, were active for 75001 and 39401 and appeared in all tests to be identical with kynurenine sulfate. It was also necessary to determine the nature of the group removed by acid hydrolysis in order to establish the structure of 31881-I. Since the α-amino group was freed by acid hydrolysis it was suspected that the mask-

ing group was either a formyl or an acetyl group. On acid hydrolysis these groups would give rise to formic and acetic acids. A 100-mg. sample of 31881-I (in 10 cc. of 1 N H_2SO_4) was hydrolyzed in a sealed glass tube placed in a boiling water bath for $1^1/_2$ hours. The tube was removed, cooled, opened and the contents poured into a distilling flask. The volatile acids were distilled over and the Duclaux distillation constants determined. The constants agreed perfectly with those found using a known

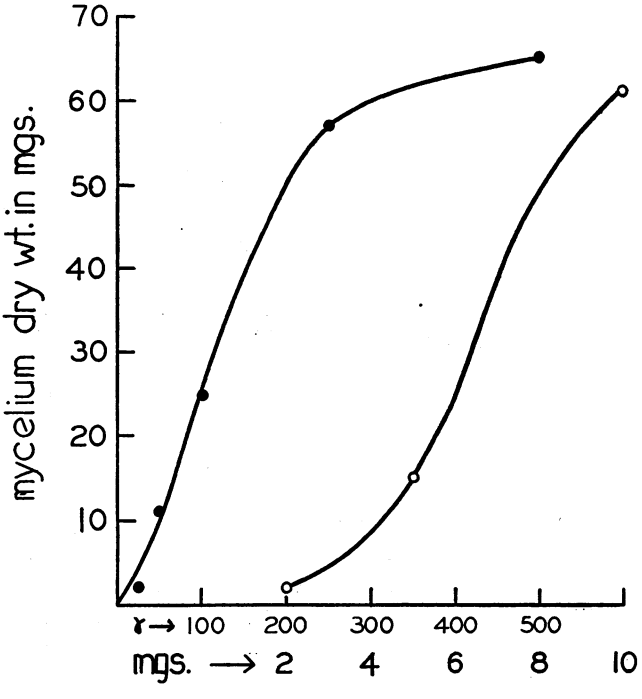


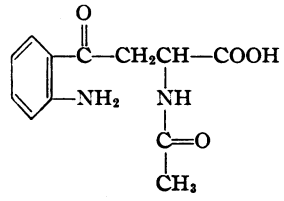
FIGURE 3

Activity of 31881-I for strain 39401 before and after acid hydrolysis. ○–○ before acid hydrolysis—mg. scale; ●–● after acid hydrolysis—γ scale.

acetic acid solution. Furthermore, 0.36 meq. of acid was present in the 100 cc. of distillate. This amount of acetic acid accounts for 90% of the theoretical amount of acid which would be liberated by the complete hydrolysis of 100 mg. of α-N-acetyl kynurenine. C-H analysis of 31881-I is compared below with that calculated for α-N-acetyl kynurenine.

	C	H
Found	57.69	5.77
Calculated for $C_{12}H_{14}O_4N_2$	57.58	5.63

On the basis of these data we have identified 31881-I as α-N-acetyl kynurenine (formula I). Final proof awaits synthesis of this compound, which is now in progress.



Formula I

To arrive at any conclusions concerning the significance of the accumulation of 31881-I it was necessary to determine whether other strains accumulate this substance. Several years ago Bonner and Beadle[17] reported the isolation of a substance (designated as 4540-II) from the filtrates of strain 4540. Because only small quantities of 4540-II were present and its activity slight, attention was focused on the more active substance accumulated by this strain, 3-hydroxy-anthranilic acid. From the data presented in their paper it can be concluded that 4540-II and 31881-I are identical. The C-H analyses of 4540-II and 31881-I are compared below:

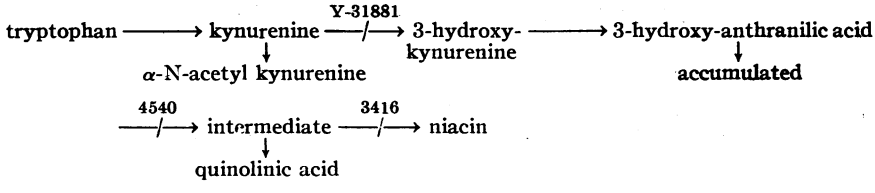
	C	H
4540-II	57.55	5.68
31881-I	57.69	5.77
Calculated for $C_{12}H_{14}O_4N_2$	57.58	5.63

Filtrates from other strains were also tested for the presence of this compound. In these experiments the standard isolation and detection procedures were employed which are reported in this paper. The strains tested were 2198 and 5256 (wild type). 31881-I could not be detected in the filtrates of either of these strains. However, this method could not be expected to detect small quantities of this substance which may be normally formed.

Discussion.—Tryptophan and kynurenine have been proposed as intermediates in the synthesis of niacin in Neurospora largely because they support the growth of one genetic type of niacin-requiring mutant strain.[1] 3-Hydroxy-anthranilic acid similarly shows niacin activity[2] but proof of its natural participation in niacin synthesis has been obtained.[5] On the basis of these observations a scheme was proposed involving tryptophan, kynurenine, 3-hydroxy-kynurenine and 3-hydroxy-anthranilic acid as precursors of niacin. Certain observations regarding the strain upon which the participation of tryptophan and kynurenine have been predicated are not readily reconcilable with the thesis that tryptophan and kynurenine serve as major niacin precursors.[5,18] However, N^{15} experiments, designed specifically to test this point, have shown conclusively

that tryptophan is the main source of niacin in Neurospora.[7] Also consistent with the proposed scheme is the fact that 3-hydroxy-kynurenine has been found active in replacing niacin.[11] However, final proof for the participation of kynurenine and 3-hydroxy-kynurenine depends upon the demonstration that these compounds can be and are synthesized by Neurospora. The work reported in this paper has shown that a metabolite of kynurenine is abnormally accumulated as a result of a genetic block in one strain. Therefore, proof has been obtained for the ability of the mold Neurospora to produce kynurenine-like compounds. Furthermore, this accumulation is associated with the inability of the strain concerned to synthesize niacin. There is a direct relationship between the two. Consistent with these observations is the fact that this mutant will grow in the presence of 3-hydroxy-kynurenine but not in the presence of kynurenine. Hence, it apparently cannot convert the latter substance to the former. Thus, it has been shown that Neurospora can and does synthesize kynurenine-like compounds and that these compounds are involved in niacin synthesis. In rats, the normal excretory product of kynurenine is either kynurenic or xanthurenic acid, depending upon the B_6 content of the diet. In view of the small quantities of α-N-acetyl kynurenine formed by strain 4540,and the larger quantities formed by strain Y-31881, it appears that this compound represents a major end-product of kynurenine metabolism in Neurospora. However, both kynurenic and xanthurenic acids might reasonably be expected to be found as kynurenine metabolites in Neurospora under certain conditions.

The following scheme represents the present status of the investigations on niacin synthesis in Neurospora. The vertical arrows indicate compounds accumulated as a result of the indicated genetic block.



Summary.—The isolation and identification of a substance possessing slight niacin activity for one mutant strain of Neurospora has been described. The strain accumulating this substance can synthesize niacin from 3-hydroxy-kynurenine or 3-hydroxy-anthranilic acid but is incapable of utilizing tryptophan or kynurenine for this purpose. If the proposed pathway from tryptophan to niacin in Neurospora is correct the accumulated compound should be kynurenine or some product of kynurenine metabolism. Such a product has been isolated and identified as α-N-acetyl kynurenine. The accumulation of this kynurenine-like compound as a result of an induced genetic block is considered direct evidence for the participation of kynurenine in the biosynthesis of niacin in Neurospora.

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THE EFFECT OF OXYGEN CONCENTRATION ON THE RATE OF X-RAY INDUCED MUTATIONS IN DROSOPHILA

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Various supplemental agents have been administered to organisms being exposed to x-rays and the effects of these agents on the induced frequency of gene mutations and chromosome aberrations have been studied. In general, the supplemental treatments which did alter the x-ray-induced mutation or chromosome rearrangement rate were found to increase this rate. However, the recent work of Thoday and Read,[1] Hayden and Smith,[2] and Giles and Riley[3] provide evidence that by lowering the oxygen tension during x-ray exposure of plant material, the induced frequency of