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DIRECT EVIDENCE FOR A TRYPTOPHAN–ANTHRANILIC ACID CYCLE IN NEUROSPORA

WILLIAM H. MATCHETT AND J. A. DEMOSS

Department of Biology, University of California, San Diego, La Jolla, Calif. (U.S.A.)

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SUMMARY

1. Experiments are described which permit a more precise understanding of the relationship between metabolism of tryptophan and accumulation of anthranilic acid in tryptophan auxotrophs of Neurospora. A cyclic interconversion of tryptophan and anthranilic acid was postulated earlier.

2. Wild type and two auxotrophic strains of Neurospora, one of which was blocked in the conversion of anthranilic acid to indoleglycerol phosphate were grown in the presence of L-[^{14}C]tryptophan labeled either in the 2-position or randomly in the benzene ring. Protein-tryptophan was isolated after growth and its specific radioactivity was compared with that of the originally supplied tryptophan. No isotopic dilution was observed in any strain grown in the presence of benzene-labeled tryptophan indicating that under these conditions endogenous synthesis of tryptophan is completely inhibited. Extensive isotopic dilution was observed in the two strains not blocked in the conversion of anthranilic acid to tryptophan when these were grown in the presence of L-[2-^{14}C]tryptophan. No significant dilution was observed in the mutant blocked in the conversion of anthranilic acid to tryptophan. These findings indicate that the postulated cycle is operating in growing mycelia.

3. Radioactive products accumulated in the growth filtrate of the blocked mutant were identified as anthranilic acid and formylanthranilic acid. The specific radioactivities of these compounds showed clearly that they were derived exclusively from exogenous tryptophan while it remained in the medium. Upon exhaustion of tryptophan, the specific radioactivity of anthranilic acid fell abruptly indicating that its continued accumulation resulted from endogenous biosynthesis via unlabeled precursors.

4. The implications of these results are discussed with reference to the earlier postulated cycle.

INTRODUCTION

Anthranilic acid is generally believed to be an intermediate in the biosynthesis of tryptophan[1]. This belief stems from the fact that among tryptophan auxotrophs of several bacteria and *Neurospora crassa* some accumulate anthranilic acid in their growth media while others are able to utilize this compound for growth.

Abbreviations: InGP, indole-3-glycerol phosphate; CDRP, 1-*o*-carboxyphenylamino-1-deoxyribulose 5-phosphate.

HASKINS AND MITCHELL[2] obtained evidence suggesting that in Neurospora some anthranilic acid arose not as a biosynthetic intermediate but as a breakdown product of tryptophan and concluded that anthranilic acid could arise in two ways in this organism. To account for their results they proposed a metabolic cycle (Fig. 1) involving tryptophan, kynurenine, anthranilic acid and an unknown precursor of

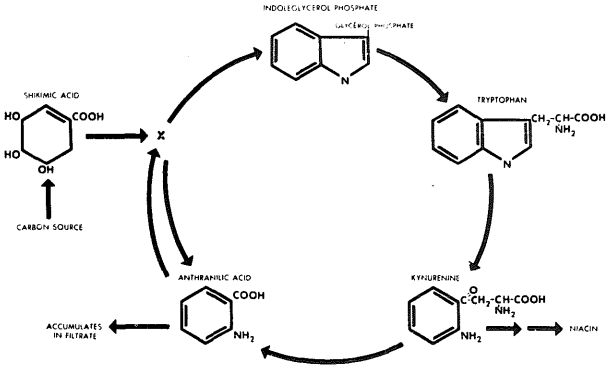


Fig. 1. The scheme of HASKINS AND MITCHELL.

InGP. In their scheme, anthranilic acid and the precursor of InGP were freely interconvertible. Thus, in their view anthranilic acid could arise either from the precursor of InGP or from the breakdown of tryptophan. Since that time evidence has accumulated which supports their view. TATUM *et al.*[3] showed that shikimic acid diluted the isotope content of anthranilic acid accumulated by a mutant of Neurospora growing in the presence of radioactive sucrose. JAKOBY[4] demonstrated that two of the three enzymes required for the conversion of tryptophan to anthranilic acid were present in extracts of Neurospora.

However suggestive of a tryptophan cycle the above evidences may be, they offer no compelling reason to believe that the proposed cycle actually operates in growing mycelia of Neurospora. The work described in this communication was carried out to obtain a more precise understanding of the relationship between the metabolism of externally supplied tryptophan and the accumulation of anthranilic acid in Neurospora. To this end Neurospora was grown in the presence of radioactively labeled tryptophan. The pattern of appearance of isotope in mycelial protein-tryptophan and in anthranilic acid and its derivatives accumulated in the growth filtrate demonstated clearly that a cycle similar to the one postulated is operating in Neurospora.

MATERIALS AND METHODS

Mutant strains 32092 (Ind-1) and 32103 (An) of *N. crassa* were maintained on slants of agar prepared from VOGEL's minimal medium[5] supplemented with 150 μg/ml

L-tryptophan. Wild type strain 74A of *N. crassa* was maintained on slants of agar prepared from the same medium containing no tryptophan. Experimental liquid cultures were inoculated with conidia obtained from slant cultures.

L-[*benzene*-$^{14}C_6$]Tryptophan was prepared from a sample of DL-[*benzene*-$^{14}C_6$]-tryptophan purchased from Nuclear Chicago Corporation. The racemic mixture was digested with tryptophanase; the indole formed was extracted with toluene and converted to L-tryptophan with a partially purified preparation of tryptophan synthetase kindly supplied by Dr. S. ENSIGN. The L-tryptophan formed was isolated from the deproteinized reaction mixture by charcoal adsorption and subsequent elution with hot 50 % ammoniacal ethanol.

L-[2-^{14}C]Tryptophan was prepared from [2-^{14}C]indole purchased from Calbiochem, by the enzymic synthesis described above.

Protein was extracted from experimental mycelia with 1.0 N NaOH, precipitated with cold 5 % trichloroacetic acid, washed with hot 5 % trichloroacetic acid and hydrolyzed with saturated barium hydroxide solution. Ba^{2+} was precipitated from the resulting hydrolyzate by the addition of dry ice. The desalted hydrolyzate was neutralized with 1.0 M potassium phosphate buffer (pH 7.8). A precipitate which formed upon addition of this buffer was removed by centrifugation. Tryptophanase was added to the hydrolyzate and the indole formed as a result of the digestion of tryptophan was extracted into toluen . Indole was determined colorimetrically[6] and its radioactivity was determined in the liquid scintillation spectrometer.

Anthranilic acid, formylanthranilic acid, and an unknown substance, hereafter called compound X for convenience, were isolated from culture filtrates by paper chromatography in methanol – butanol – benzene – H_2O (2:1:1:1, v/v) ascending. Formylanthranilic acid and compound X were converted to anthranilic acid by acid hydrolysis (0.2 N HCl at 90° for 5 min). The anthranilic acid was then rechromatographed in a second solvent system; isopropanol – ammonia – H_2O (100:5:10, v/v) ascending. The spots obtained on the second chromatogram were eluted with hot absolute ethanol and the eluates assayed spectrofluorimetrically for anthranilic acid. The eluates were assayed for radioactivity in the liquid scintillation spectrometer. Identity of anthranilic acid and formylanthranilic acid was confirmed by cochromatography with authentic compounds and by comparison of their activation and fluorescence spectra with those of authentic compounds.

Measurement of radioactivity was carried out with a liquid scintillation spectrometer. L-Tryptophan was assayed as indole dissolved in toluene. Anthranilic acid, formylanthranilic acid and compound X were assayed as anthranilic acid dissolved in ethanol. Aliquots of these solutions were placed in vials containing 10 ml of scintillation fluid (per litre toluene, 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene). Samples were counted ten minutes at a counting rate of approx. 500 counts/min. The counting efficiency was about 60 % and quenching for all determinations was insignificant.

RESULTS

If growing mycelia of Neurospora are able to catalyze the reactions necessary to convert tryptophan to anthranilic acid at an appreciable rate and if this conversion is in fact being carried out *in vivo*, then a relatively simple experiment would suffice

to demonstrate the phenomenon. Strains whose growth is supported by anthranilic acid as well as by indole or tryptophan should give more growth per μmole of tryptophan taken up from the medium than strains whose growth is supported only by indole or tryptophan. The growth of wild type, which is tryptophan independent should not be influenced by these reactions. The data obtained in such an experiment are presented in Table I. Ind-1 is a strain blocked in the conversion of anthranilic acid to CDRP[7]. An is a strain blocked in the conversion of shikimic acid to anthranilic acid.

TABLE I

GROWTH YIELD OF WILD TYPE AND TWO TRYPTOPHAN AUXOTROPHS IN MINIMAL MEDIUM SUPPLEMENTED WITH TRYPTOPHAN

Conidia were inoculated in 15 ml minimal medium supplemented with 0.75 μmole/ml L-tryptophan. Cultures were incubated 96 h with constant shaking in a water bath maintained at 20°.

Strain	*Dry weight* (mg)*
Ind-1	21, 22
An	87, 84
Wt	86, 84

* Cultures were filtered through Whatman No. 1 filter paper, the mycelia were quickly frozen and then lyophilized. The dried mycelial pads were then weighed on an analytical balance. Values given are those observed in duplicate cultures of each strain.

Tryptophan had disappeared from the medium of each culture after fifty hours' incubation. That Ind-1 gave one-fourth the amount of mycelium produced by An per μmole of tryptophan removed from the medium suggests that tryptophan is being converted to products which will not support the growth of Ind-1 but which will support the growth of a mutant requiring anthranilic acid.

These results also suggest that the reactions converting tryptophan to anthranilic acid coupled to the reactions forming tryptophan from anthranilic acid constitute a cyclic interconversion of tryptophan and anthranilic acid. If such a cycle is operating in Neurospora then carbon atoms not included in the benzene ring of the tryptophan molecule would be lost in one complete turn of the cycle. Table II shows the results

TABLE II

SPECIFIC RADIOACTIVITY OF TRYPTOPHAN ISOLATED FROM MYCELIAL PROTEIN AFTER GROWTH IN L-[2-^{14}C]TRYPTOPHAN AND L-[BENZENE-$^{14}C_6$]TRYPTOPHAN

Conidia inoculated into 15 ml minimal medium supplemented with 0.75 μmole/ml L-[2-^{14}C]-tryptophan of specific radioactivity 376 mμC/μmole or with L-[*benzene*-^{14}C]tryptophan of specific radioactivity 40 mμC/μmole. Cultures were incubated and harvested as indicated in Table I.

Strain	*Specific radioactivity of protein-tryptophan* (mμC/μmole tryptophan)*	
	L-[2-^{14}C]tryptophan	*L-[benzene-$^{14}C_6$]-tryptophan*
Ind-1	298, 326	35.8
An	74, 86	39.2, 39.8
Wt	87, 89	39.0, 41.6

* See METHODS. Values presented are those obtained from duplicate cultures of each strain.

of an experiment in which the two mutant strains and wild type were grown in the presence of L-[^{14}C]tryptophan labeled either in the 2 position of the indole nucleus or uniformly in the benzene ring. The results obtained with tryptophan labeled in the benzene ring indicate that under the conditions employed essentially no tryptophan of endogenous origin is incorporated into mycelial protein. The results obtained with cultures supplemented with L-[2-^{14}C]tryptophan show clearly that in the An and Wt strains the 2 position of the tryptophan molecule is extensively diluted but is only very slightly diluted in the Ind-1 strain. These results suggest that in the two strains (Wt and An) which are not blocked in the conversion of anthranilic acid to tryptophan the cycle is indeed turning. In the mutant strain having a block within the cycle (Ind-1) dilution of the 2 position of tryptophan does not occur to any appreciable extent since anthranilic acid derived from tryptophan cannot be reconverted to tryptophan.

The data of this experiment permit a calculation of the total amount of tryptophan actually incorporated into protein (*i.e.*, specific radioactivity of protein/specific radioactivity of tryptophan = μmoles tryptophan/mg protein). When this calculation was made from pooled data of several experiments of the type described in Table II, a value of 0.1 μmole tryptophan/mg mycelial protein was obtained. Table I shows that a 15-ml culture supplemented with 11.2 μmoles of tryptophan yields about 20 mg of Ind-1 mycelia. About one-fourth of the dry weight of mycelia cultured under these conditions consists of protein. Thus, of the 11.2 μmoles of tryptophan originally supplied, only about 0.5 μmole is actually incorporated into mycelial protein. Since tryptophan disappears from the medium of such a culture after about 50 h of incubation, it is clear that 95 % of the exogenously supplied tryptophan is unavailable for protein synthesis and apparently undergoes degradation.

The results of an experiment designed to illustrate the fate of the carbon skeleton of exogenously supplied tryptophan during and after its disappearance from the growth medium of Ind-1 are presented in Figs. 2 and 3. Conidia of Ind-1 were inoculated into 30 ml of minimal medium supplemented with 0.875 μmole/ml L-[*benzene*-$^{14}C_6$]tryptophan. The culture was incubated at 20° with constant shaking. Samples of the medium were withdrawn periodically during the 100-h growth period. Tryptophan remaining in the medium, specific radioactivity of tryptophan and total radioactivity of the medium are plotted in Fig. 2a. The fact that total radioactivity remained essentially constant throughout the culture period shows that tryptophan taken up by the mycelia is almost quantitatively converted to breakdown products which are excreted into the medium as fast as they are formed.

Paper chromatography of the growth filtrate samples revealed that essentially all of the radioactivity was present in three main spots. The blue fluorescence, positive Ehrlich test and R_F values of these spots were suggestive of anthranilic acid or derivatives of anthranilic acid. Two of these three spots were subsequently identified as anthranilic acid and formylanthranilic acid (see METHODS). The third spot, compound X, has been provisionally identified as a glycosylamine of anthranilic acid. Cochromatography of isolated compound X and N-*o*-carboxyphenyl-D-ribosylamine, prepared by the method of DOY AND GIBSON[8] revealed that both compounds migrated as a single spot in the butanol–benzene–methanol–water solvent. Both compounds exhibited activation peaks at 335 mμ and fluorescence peaks at 410 mμ as determined with an Aminco-Bowman spectrofluorimeter. The synthesized compound gave the

pink color characteristic of ribose and other pentoses with commercially prepared aniline hydrogen phthalate spray reagent. Compound X, however, did not. A spot having many of the characteristics of compound X formed spontaneously when anthranilic acid and either ribose or glucose were applied together to a chromatogram. Upon hydrolysis with acid compound X yielded anthranilic acid and a material giving a positive aniline hydrogen phthalate test typical of glucose. These observations suggested the possibility that compound X was formed spontaneously in the process of applying filtrate samples to the chromatographic papers. In any case, it was possible to isolate the anthranilic acid-moiety of compound X and to determine its specific radioactivity.

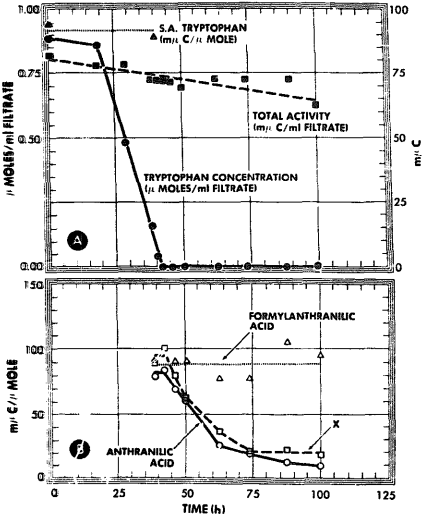
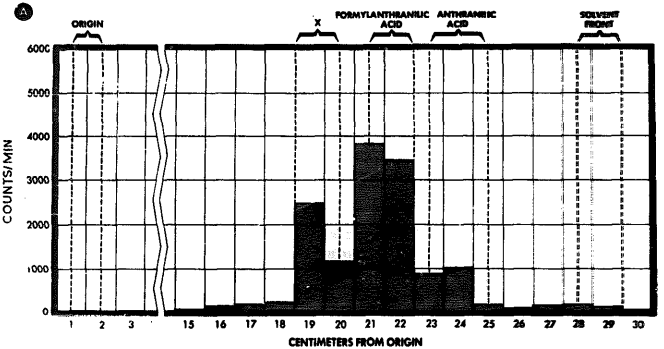


Fig. 2. (A) Tryptophan disappearance, specific radioactivity of tryptophan and total radioactivity remaining in filtrate plotted as a function of age of culture. Tryptophan was determined by the method of DEMOSS[29]. Specific radioactivity of tryptophan was determined by assaying an aliquot of the indole formed from the tryptophanase digestion of tryptophan in the liquid scintillation spectrometer. Total radioactivity remaining in the filtrate was determined by assaying an aliquot in the scintillation spectrometer, and is reported as mμC/ml filtrate. (B) Specific radioactivities of the three major accumulation products plotted as a function of the age of the culture. Formylanthranilic acid and compound X were converted to anthranilic acid and specific radioactivity of this substance was determined as indicated in METHODS.

Fig. 2b shows specific radioactivity of the three major radioactive products accumulated in the medium. Formylanthranilic acid remained at the same specific radioactivity as the original tryptophan throughout the culture period. Anthranilic acid and the anthranilic moiety of compound X, on the other hand, were in isotopic

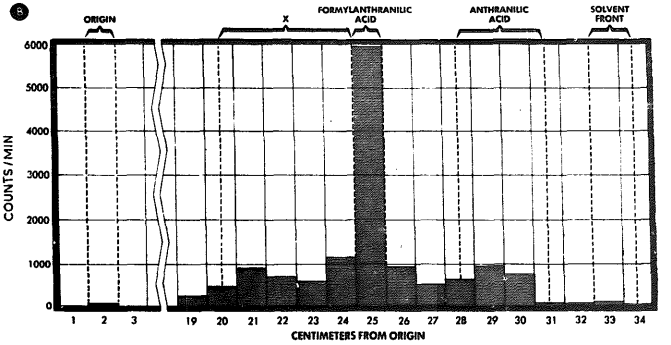


	PERCENT TOTAL ACTIVITY	TOTAL ACTIVITY OF EACH COMPOUND	SPECIFIC ACTIVITY	μMOLES/ml FILTRATE
X	23	17 mμC	105 mμC/μMOLE	0.16
FORMYLANTHRANILIC ACID	54	39 mμC	77 mμC/μMOLE	0.49
ANTHRANILIC ACID	15	11 mμC	85 mμC/μMOLE	0.13

equilibrium with tryptophan only while it remained in the medium. When tryptophan was exhausted from the medium, the specific radioactivities of these compounds fell rapidly. The fact that the specific radioactivity of formylanthranilic acid was not diminished after the disappearance of tryptophan from the medium shows clearly that this compound precedes anthranilic acid in the metabolic sequence degrading tryptophan, and that the step from formylanthranilic acid to anthranilic acid is an irreversible one. Furthermore, when Neurospora was grown in the presence of L-[2-^{14}C]tryptophan, radioactive formylanthranilic acid accounted for all the filtrate radioactivity and over 50 % of the total radioactivity originally supplied.

Fig. 3 shows radioactivity profiles of chromatograms of filtrate samples taken (a) at 44 h (b) at 100 h. These data show the quantitative significance of the three compounds whose specific radioactivities are plotted in Fig. 2B. At 44 h, 92 % of the filtrate radioactivity was present in these three compounds and at 100 h 85 % of the filtrate activity was still present in these compounds.

It was shown above that only about 5 % of the exogenously supplied tryptophan is actually incorporated into mycelial protein. The data of Figs. 2 and 3 show that the remaining 95 % of the originally supplied tryptophan is converted to anthranilic acid or its equivalent. At 44 h (the time of tryptophan exhaustion from the medium) 0.87 μmole/ml tryptophan had disappeared from the growth filtrate and the equivalent of 0.78 μmole/ml of anthranilic acid had accumulated. The specific radioactivity of the originally supplied tryptophan was 91 mμC/μmole. The average specific radioactivity of anthranilic acid accumulated was 89 mμC/μmole. These facts demonstrate clearly that tryptophan was the sole source of all the anthranilic acid or its equivalent



	PERCENT TOTAL ACTIVITY	TOTAL ACTIVITY OF EACH COMPOUND	SPECIFIC ACTIVITY	μ MOLES/ml FILTRATE
X	27	17 mμ C	19 mμ C/μ MOLE	0.89
FORMYLANTHRANILIC ACID	41	25.5 mμ C	100 mμ C/μ MOLE	0.25
ANTHRANILIC ACID	17	11 mμ C	10 mμ C/μ MOLE	1.1

Fig. 3. Radioactivity profiles of streak chromatograms in butanol–benzene–methanol–water (2:1:1:1, v/v, ascending) of filtrate samples obtained in the experiment described in Fig. 2. A, 44-h sample; B, 100-h sample. Chromatograms were prepared by applying 0.5 ml of culture filtrate in a streak 3 inches in length at the origin. Positions of the compounds separated were located with ultraviolet light and are indicated by brackets above the histograms. A strip 1 inch wide and extending from 1 cm beneath the origin to 2 cm above the solvens front of each chromatogram was cut in pieces each 1 cm in width. Each piece of paper was placed in a scintillation vial and its radioactivity determined. The total counts associated with any one spot divided by the total counts observed in the entire strip equals the percent of the total filtrate radioactivity associated with the compound. Total radioactivity associated with the compound divided by its specific radioactivity equals the amount of the compound present in the filtrate. For total filtrate radioactivity at 44 h and 100 h, see Fig. 2a.

accumulated while tryptophan remained in the medium. These data also illustrate that the presence of tryptophan completely inhibits the synthesis of anthranilic acid from the carbon source. Fig. 3B shows that dilution of anthranilic acid and anthranilic acid liberated from compound X occurs as a result of biosynthesis of this compound from unlabeled precursors since the 5–10 fold reductions in specific radioactivity are accompanied by 5–10 fold increases in the amounts of this compound found in the filtrate at 100 h.

DISCUSSION

The results obtained in this investigation provide an answer to the question concerning the sources of anthranilic acid accumulated in culture filtrates of tryptophan auxotrophs of Neurospora. They show clearly that this compound is derived almost exclusively from the degradation of externally supplied tryptophan while this amino

acid remains in the medium. Anthranilic acid synthesized after the exhaustion of tryptophan from the medium must derive from unlabeled precursors, in all likelihood from the carbon source via shikimic acid. The present data therefore permit a more precise interpretation of the results of HASKINS AND MITCHELL[2] and those of TATUM *et al.*[3]. Although direct evidence that anthranilic acid was derived from tryptophan was not adduced by HASKINS AND MITCHELL[2], their conclusions were quite correct. TATUM *et al.*[3] were also quite correct in their conclusion that anthranilic acid can arise from the carbon source via shikimic acid, although they did not rule out the possibility that anthranilic acid might, in their case, arise from the carbon skeleton of the externally supplied indole via cycling of tryptophan formed from it.

With the single exception of tryptophan pyrrolase, all of the enzymes necessary for catalyzing the reactions of a tryptophan–anthranilic acid cycle have been demonstrated in extracts of Neurospora[7,9–11]. On the basis of these observations and those of the present study the cycle may be written as indicated in Fig. 4. The relationship of enzymes and substrates in that portion of the cycle concerned with the conversion of formylkynurenine to anthranilic acid is of particular interest since two routes for this sequence of reactions are possible. Formylkynurenine can serve as a substrate for kynureninase and kynurenine formamidase: the former giving rise to formylanthranilic acid and the latter giving rise to kynurenine. Kynureninase can act upon either of two substrates, namely, formylkynurenine or kynurenine. Likewise, kynurenine formamidase can act upon either of two substrates, namely, formylkynurenine and formylanthranilic acid.

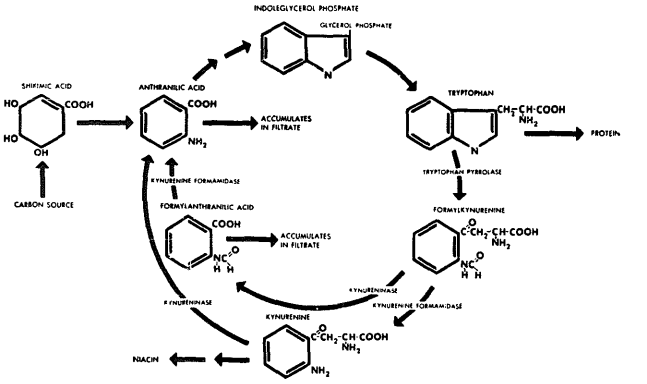


Fig. 4. The tryptophan–anthranilic acid cycle.

The observation that formylanthranilic acid accounts for most of the radioactivity present in the filtrate immediately after the disappearance of tryptophan (Fig. 3A) is consistent with JAKOBY's[4] results. He found that in crude extracts of Neurospora, grown under conditions similar to those of the experiment of Figs. 2 and 3, the specific activity (mμmoles of substrate converted/h/mg protein) of kynureninase was 10-fold

higher than the specific activity of kynurenine formamidase. He also measured Michaelis constants of each of these enzymes for formylkynurenine and found that kynureninase had about 100 times the affinity for this substrate as that exhibited by kynurenine formamidase. These facts suggest that, under these conditions, the reaction of greatest quantitative significance catalyzed by kynureninase is the conversion of formylkynurenine to formylanthranilic acid. It would thus appear that the major flow of carbon from tryptophan to anthranilic acid proceeds via formylkynurenine and formylanthranilic acid and not via kynurenine[1,12,13].

On the other hand, it is well-known that kynurenine is an intermediate in the biosynthesis of niacin[1]. Hence, kynurenine formamidase must catalyze the conversion of formylkynurenine to kynurenine at least to a limited extent. It is quite likely that the adaptive response exhibited by kynureninase[12] in the presence of external tryptophan plays a significant role in the regulation of these alternate pathways. In any case, it is clear that this system constitutes an excellent model for studying mechanisms regulating the metabolism of small molecules.

That externally supplied tryptophan completely inhibits incorporation of endogenously synthesized tryptophan into protein is shown clearly in the data of Table II. It has already been shown that the presence of tryptophan represses formation of tryptophan synthetase[14,15] and other enzymes in the biosynthetic pathway leading to tryptophan[16]. Repression, however, was observed only in auxotrophic strains requiring anthranilic acid, indole or tryptophan for growth. The levels of repression of enzyme formation observed in these cases and the fact that repression could not be demonstrated in prototrophically growing strains suggest that the present observation (Table II) is a manifestation of another controlling factor in tryptophan biosynthesis, namely end-product inhibition of the biosynthetic sequence. The data of Fig. 2B indicate that the site of action of tryptophan as a feedback inhibitor is a step prior to the formation of anthranilic acid. This observation is certainly consistent with the results obtained in *E. coli*[17] and with the general concept of end-product inhibition of the action of a biosynthetic sequence[18]. The fact that synthesis of anthranilic acid from unlabeled precursors starts immediately after the disappearance of tryptophan from the medium suggests strongly that this is indeed a case of inhibition of the action of an enzyme rather than absence of the system.

The demonstration, in this work, that an exogenously supplied required growth supplement can be converted by a series of reactions to a compound which behaves as an intermediate in the biosynthetic sequence leading to the required compound suggests that data on accumulations should be interpreted cautiously.

ACKNOWLEDGEMENTS

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