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The Relation between Biotin and Tryptophan Metabolism Studied in *Neurospora crassa*

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(Received 22 March 1954)

The observation by Krehl, Teply, Sarma & Elvehjem (1945) that rats deficient in nicotinic acid would grow well if tryptophan was supplied elicited much interest, and a number of reports have appeared showing that tryptophan is the dietary precursor of nicotinic acid and its derivatives in the rat (Rosen, Huff & Perlzweig, 1946), pig (Luecke, McMillen, Throp & Tull, 1947), horse (Schweigert, Pearson & Wilkening, 1947), dog (Singal, Sydenstricker & Littlejohn, 1948) and man (Saret & Goldsmith, 1947). It has also been established both in the rat (Heidelberger, 1949) and the Ascomycete fungus, *Neurospora* (Mitchell & Nyc, 1948) that tryptophan is converted into nicotinic acid through a series of intermediary substances, such as kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid.

Some of the vitamins of the B group influence the conversion of tryptophan into nicotinic acid. The investigations of Lepkovsky, Roboz & Haagen-Smit (1943), Schweigert & Pearson (1947), Rosen, Huff & Perlzweig (1947) showed that pyridoxine has a role in the conversion of tryptophan into nicotinic acid in the rat, mice and swine. Junqueira & Schweigert (1948) noted reduced urinary excretion of nicotinic acid and *N'*-methylnicotinamide in rats deficient in thiamine, riboflavin, pyridoxine and pteroylglutamic acid. Porter, Clark & Silber (1948) showed that thiamine, riboflavin and pyridoxine also affect the excretion of kynurenine, kynurenic acid and xanthurenic acid. Henderson, Weinstock & Ramasarma (1951) reported that during riboflavin and pyridoxine deficiencies, the ability of the rat to convert tryptophan and kynurenine into quinolinic acid was markedly impaired. These deficiencies, however, had no effect on the conversion of 3-hydroxyanthranilic acid into quinolinic acid.

Shanmuga Sundaram, Ranganathan & Sarma (1951), using deoxypyridoxine, an antimetabolite of pyridoxine, showed that pyridoxine is involved in the biosynthesis of nicotinic acid in germinating pulses (*Phaseolus mungo*).

The vitamin biotin (I) has been shown to be involved in amino acid oxidation (Lichstein & Umbreit, 1947; Thayer & Horowitz, 1951), and since this process takes place in tryptophan metabolism (Dalglish, 1951) it was thought that biotin might possibly have an influence in the metabolism of tryptophan as well. Further, Shanmuga Sundaram & Sarma (1954) found that the antimetabolite of biotin, γ -(3:4-ureylenecyclohexyl)butyric acid

(II), exerts a deleterious effect on the biosynthesis of nicotinic acid in germinating pulses and this was accompanied by a marked accumulation of tryptophan. This inhibition of the synthesis of nicotinic acid and utilization of tryptophan was overcome by the addition of excess biotin. However, it was found that certain pyridine nucleotide-linked dehydrogenases of *Aspergillus oryzae* were considerably affected during biotin deficiency (Tirunarayanan & Sarma, to be published). Hence, studies were

In vitro studies. The organism *N. crassa*, grown on a medium containing L-tryptophan for 72 hr., was used. The wet mycelium was washed thoroughly and made into a uniform suspension in ice-cold 0.1M phosphate buffer (pH 6.8) using a Waring Blender. A known volume of the suspension was added to (i) L-tryptophan, (ii) L-tryptophan with the ureylene compound and (iii) L-tryptophan, the ureylene compound and excess biotin. After 16 hr. incubation at 37°, the reaction was stopped using trichloroacetic acid, and tryptophan in the solution was estimated immediately.

Estimation of tryptophan. Tryptophan in the incubated solution was estimated colorimetrically using the methods of Horn & Jones (1945) and Eckert (1943).

RESULTS AND DISCUSSION

The results presented in Figs. 3 and 4 show that the antimetabolite of biotin only affects the utilization of tryptophan; the inhibition in utilization is overcome by biotin, as represented in Table 1. As the ureylene compound has not any significant influence on the utilization of the other metabolites of tryptophan $\rightarrow$ nicotinic acid mechanism in the two strains of the fungus, it is evident that biotin has a role only in the conversion of tryptophan into the primary intermediate kynurenine.

In their preliminary work on the tryptophan $\rightarrow$ kynurenine conversion, Knox, Mero, Grossman & Auerbach (1949) first showed that kynurenine formation from tryptophan in liver homogenates of rats is carried out only under aerobic conditions. Later, Knox & Mehler (1950) and Mehler & Knox (1950) found that tryptophan is converted by the supernatant liquid of a fresh liver homogenate into a diazotizable, aromatic amine, identified as formylkynurenine, with the uptake of one molecule of oxygen and simultaneous liberation of hydrogen

peroxide. The formylkynurenine so formed is broken down by a second enzyme, called 'formylase' into kynurenine and formic acid.

The first reaction, involving the formation of formylkynurenine from tryptophan, was shown by Knox & Mehler (1950) to consist of two steps; tryptophan is first converted by peroxide under the action of 'peroxidase' into an unknown intermediate *A*, and this is followed by a second coupled reaction in which *A* is oxidized to formylkynurenine by an 'oxidase' with the formation of hydrogen peroxide. The scheme of reactions can be represented as follows:

Fig. 4. Effect of γ -(3:4-urelenecyclohexyl)butyric acid on the utilization of: (a) DL-kynurenine sulphate for strain 39401; (b) I and II, DL-3-hydroxykynurenine for strains 39401 and 5029, respectively; (c) I and II, 3-hydroxyanthranilic acid for strains 39401 and 5029 respectively; (d) I and II, nicotinic acid for strains 39401 and 5029 respectively. Each flask contained 5 ml. basal medium of double concentration of the metabolites needed for maximal growth and the inhibitor was added at varying concentrations as solution in water to make the final volume 10 ml.

Fig. 3. Effect of γ -(3:4-urelenecyclohexyl)butyric acid on the utilization of: I, L-tryptophan; II, DL-formylkynurenine by *Neurospora crassa* (39401). Each flask contained 5 ml. basal medium of double concentration with the metabolites needed for maximal growth and the inhibitor was added at varying concentrations as solutions in water to make the final volume 10 ml.

Table 1. Influence of biotin on tryptophan utilization by *Neurospora crassa* (39401)

In all experiments, 5 ml. of basal medium of double concentration were taken and the substances were added as aqueous solutions to make the final volume 10 ml. After sterilization and inoculation, incubated at 30° for 72 hr. The experiment was repeated 4 times with a duplicate each time.

Supplement	Weight of mycelium (mg.)
L-Tryptophan (200 μ g.)	20.0
L-Tryptophan (200 μ g.) + ureylene compound (2 mg.)	4.5
L-Tryptophan (200 μ g.) + ureylene compound (2 mg.) + biotin (3 μ g.)	18.4

Table 2. *Degradation of tryptophan by Neurospora mycelia suspension in presence of ureylene compound and biotin*

In all experiments, 9 mg. of L-tryptophan in 3 ml. water were treated with 4 ml. *Neurospora* suspension in buffer; 1 ml. suspension contained 12 mg. dry wt. To this medium additions were made as shown in column 1 and incubated for 16 hr. at 37°. The reaction was stopped by adding 1 ml. of 10% trichloroacetic acid and tryptophan was estimated immediately. The experiment repeated 3 times with duplicate determinations.

Supplement	Amount of tryptophan (mg.)		Decrease in tryptophan (mg.)
	Horn & Jones's (1945) method	Eckert's (1943) method	
Buffer (5 ml.)	6.2	6.0	2.8-3.0
Ureylene compound (4 mg.) in buffer (5 ml.)	8.5	8.4	0.5-0.6
Ureylene compound 4 mg. + biotin 25 µg. in 5 ml. buffer	5.4	5.8	3.2-3.6

Since the ureylene derivative has no influence on the utilization of formylkynurenine, as may be observed from the figures, it is clear that biotin is involved in one or both of the enzyme systems, peroxidase or oxidase, which take part in the formation of formylkynurenine. Although the identity of the unknown intermediate *A* is open to question, Dalglish, Knox & Neuberger (1951) suggested that peroxide adds on to tryptophan to give 2:3-dihydro-2:3-dihydroxytryptophan, which by dehydrogenation with simultaneous ring opening could yield formylkynurenine. The verification of this attractive suggestion must necessarily await the isolation or the synthesis of the diol. The results obtained by incubating tryptophan with the mycelial suspension (Table 2) show that only about 6% of tryptophan is oxidized in presence of the ureylene compound compared with about 35% in the control experiment. The addition of biotin completely eliminates the deleterious effect of the ureylene compound. The fact that tryptophan is apparently unchanged, as shown by the chemical estimation, indicates that biotin may influence the peroxidase step as it does the oxidative deamination of various amino acids in *N. crassa* (Bender, Krebs & Horowitz, 1949; Burton, 1951; Siva Sankar, Tirunarayanan & Sarma, 1952). However, the manner in which biotin influences the oxidation of tryptophan can be determined only after investigating whether there is any formation of an unknown intermediate compound with an absorption in the ultraviolet different from that of tryptophan and formylkynurenine or with different physical or chemical characteristics. Such a study is in progress.

SUMMARY

1. γ -(3:4-Ureylencyclohexyl)butyric acid, an antimetabolite of biotin, inhibits the utilization of tryptophan by a nicotinic acid-requiring mutant of *N. crassa* (39401) and this inhibition is overcome by the addition of biotin. This indicates that biotin is involved in the metabolism of tryptophan.

2. Biotin functions only in the tryptophan $\rightarrow$ formylkynurenine reaction and the utilization of formylkynurenine is not affected by biotin deficiency.

3. The ureylene derivative does not affect the utilization of other intermediates in the conversion of tryptophan into nicotinic acid such as formylkynurenine, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid and nicotinic acid.

4. *In vitro* studies with suspensions of *Neurospora mycelia* show an accumulation of tryptophan during biotin deficiency, and it is suggested that biotin may be involved in the formation of an unknown intermediate between tryptophan and formylkynurenine.

The authors wish to express their grateful thanks to Dr W. E. Knox for the generous gift of DL-formylkynurenine, Dr C. P. Berg for DL-kynurenine sulphate, Dr C. E. Dalglish and Dr B. S. Schweigert for 3-hydroxyanthranilic acid, Professor A. Butenandt for 3-hydroxykynurenine and Dr R. O. Roblin (jun.) for the γ -(3:4-ureylencyclohexyl)-butyric acid used in this investigation.

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Biochemistry of Fluoroacetate Poisoning: the Effect of Fluorocitrate on Purified Aconitase

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(Received 20 April 1954)

The toxic effect of fluoroacetate has been proved to be due to the enzymic synthesis of fluorocitrate (Peters, 1952; Peters, Wakelin, Buffa & Thomas, 1953). The hypothesis was advanced that fluorocitrate leads to the accumulation of citrate in animal tissues as a result of the inhibition of aconitase (Lotspeich, Peters & Wilson, 1952). Peters & Wilson (1952), working with impure fluorocitrate and crude aconitase preparations, produced tentative evidence that the inhibition was competitive. In order to establish this point the experiments of Peters & Wilson (1952) were repeated and extended using the highly purified, stable aconitase preparation of Morrison (1954). A comparison was also made of the effects of the enzymically prepared and synthetic fluorocitrate upon the enzyme.

The conclusion that the inhibition of aconitase by enzymically prepared fluorocitrate is competitive has been confirmed; at the same time interesting differences in the action of the two fluorocitrate preparations on aconitase have appeared. For a preliminary communication, see Morrison & Peters (1954).

EXPERIMENTAL

Preparation and activation of aconitase. Aconitase was prepared and activated in the presence of 5×10^{-4} M-Fe²⁺ and 10^{-2} M cysteine as described by Morrison (1954). The

enzyme solutions contained between 30 and 50 μ g. of the final aconitase preparation/ml.

Formation of cis-aconitate. Aconitase activity was determined by following the formation of *cis*-aconitate from *isocitrate* at 240 m μ . by the method of Racker (1950). It was found that when aconitase, activated with Fe²⁺ and cysteine, was added to both citrate and *isocitrate* there was a rapid initial increase in the extinction coefficient. With *isocitrate* as substrate, this rapid increase continued over a period of about 1 min., after which the reaction rate became linear. With citrate as substrate, the reaction rate became linear after about 2 min. This rapid increase in the extinction coefficient was a non-enzymic reaction, for when Fe²⁺ and cysteine were added to citrate or *isocitrate* in the same concentrations in which they were present in the enzyme solution, identical rapid increases in the extinction coefficient were obtained. At the point where the reaction rate in the presence of the enzyme became linear, the non-enzymic reaction ceased. In view of this finding, it was important to distinguish the non-enzymic from the enzymic reaction rate. Only the enzymic reaction rates have been plotted in the graphs.

Reagents. Fluorocitrate synthesized enzymically will be referred to as 'natural' fluorocitrate. Samples were prepared by the method of Peters, Wakelin, Buffa & Thomas (1953). Specimens of synthetic fluorocitrate (Rivett, 1953) were given by Dr D. E. A. Rivett. Both preparations had been standardized on kidney particles by the method of Buffa, Peters & Wakelin (1951).

Trichloromethyl paraconic acid was prepared by the method of Fittig & Miller (1889) and converted into DL-*isocitric* lactone by the method of Krebs & Eggleston (1944). Sodium *isocitrate* was formed by hydrolysis of the lactone with NaOH as described by Krebs & Eggleston (1944). L-Cysteine hydrochloride was obtained from Roche Products Ltd. All other reagents were A.R.

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