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End-Product Regulation of the Tryptophan-Nicotinic Acid Pathway in *Neurospora crassa*

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The regulation of the tryptophan-nicotinic acid pathway in *Neurospora crassa* was examined with mutants (*nic-2*, *nic-3*) which require nicotinamide for growth. The accumulation of *N*-acetylkynurenin and 3-hydroxyanthranilic acid by these mutants served to estimate the level of function of the early reactions in the pathway. In still cultures, maximal accumulation occurred with media containing growth-limiting amounts of nicotinamide; the accumulation of intermediates was almost negligible with nicotinamide in excess. Only nicotinamide and closely related compounds which also supported the growth of these mutants inhibited the accumulation of intermediates. The site of inhibition was assessed to be between tryptophan and kynurenin (or *N*-acetylkynurenin). The synthesis of *N*-acetylkynurenin was examined in washed germinated conidia suspended in buffer; the level of *N*-acetylkynurenin-synthesizing activity was inversely related to the concentration of nicotinamide in the germination medium. The addition of large amounts of nicotinamide to suspensions of germinated conidia did not affect their *N*-acetylkynurenin-synthesizing activity. Formamidase activity, kynurenin-acetylating activity, and gross tryptophan metabolism in germinated conidia was not influenced by the concentration of nicotinamide in the germination medium. The results obtained indicate that the site of inhibition by nicotinamide is the first step in the pathway, the tryptophan pyrrolase reaction. The data are interpreted as nicotinamide or a product thereof, such as nicotinamide adenine dinucleotide, acting as a repressor of the formation of tryptophan pyrrolase in *N. crassa*.

It is now generally recognized that biosynthetic reaction sequences may be quantitatively regulated by the end product of such sequences acting either as an inhibitor of an early reaction or as in the repression of the formation of the enzymes involved, or both. Studies of end-product regulation have centered largely on the biosynthesis of amino acids and purines and pyrimidines, but relatively little work has been done on the regulation of the biosynthesis of vitamins or coenzymes. Only in the case of nicotinic acid biosynthesis has the probable operation of end-product feedback control been demonstrated; the first enzyme in the tryptophan-nicotinic acid pathway, tryptophan pyrrolase, in purified preparations from *Xanthomonas pruni* (31) and rat liver (6) has been shown to be noncompetitively inhibited by reduced nicotinamide adenine dinucleotide phosphate and certain related compounds.

The pathway of biosynthesis of nicotinic acid and its derivative coenzymes from tryptophan has been long recognized in *Neurospora crassa* (2, 26), mammals (11, 24), and *X. pruni* (7, 32), and more recently it has been demonstrated to occur in *Streptomyces* (20) and in aerobically grown *Saccharomyces cervisiae* (1). The first steps in the tryptophan-nicotinic acid pathway appear to be inducible. Tryptophan and hydrocortisone stabilize or activate and induce the formation of tryptophan pyrrolase in rat liver (14, 28); tryptophan coordinately induces tryptophan pyrrolase, kynurenin formamidase, and kynureninase in *X. pruni* (4); *N*-formylkynurenin induces kynureninase in *N. crassa* (29). Similar enzymes are induced by tryptophan in bacterial species which do not use tryptophan as a precursor of nicotinic acid (25, 27). Tryptophan pyrrolase activity has not been obtained in cell-free preparations of *Neurospora* or *Saccharomyces* (1). The above observations with in vitro preparations represent only a few of the possible sites and types of regulation which may occur in vivo in the biosynthesis of nicotinic acid and nicotinamide coenzymes (5), and they encompass only a few organisms.

In this study the early steps in the tryptophan-nicotinic acid pathway in *N. crassa* are examined

in vivo as possible sites of regulatory activity. Advantage is taken of nicotinic acid-requiring strains which accumulate intermediate compounds in the pathway; these compounds are used as indicators of the level of activity of the reactions leading to their synthesis. A similar technique has been used to investigate in vivo aspects of the regulation of the biosynthesis of tryptophan in *N. crassa* (16, 17, 19). The results of the present study suggest that nicotinamide (or products thereof) inhibits or represses the formation of only tryptophan pyrrolase activity.

MATERIALS AND METHODS

Neurospora strains. N. crassa 74A was used as a source of kynureninase. Strain Y-31881 (*nic-3*) was used for the production of α-*N*-acetylkynurenin (NAK; see reference 33); this strain also requires inositol. Strain 4540 (*nic-2*) accumulates 3-hydroxy-anthranilic acid (3) which was used as an indicator for the functioning of the tryptophan-nicotinic acid pathway to that point. Strain RC-53-2A (*nic-3, kyn*) is a double mutant obtained from a cross between a *nic-3* derivative of strain Y-31881 and a mutant, produced in this laboratory, which is noninducible for kynureninase; this strain accumulates NAK and has a low-efficiency tryptophan-anthranilate cycle (10, 23), since it can form only very low levels of kynureninase. Strain RC-53-2A was used to estimate the activity of the tryptophan-nicotinic acid sequence to kynurenin.

Media and culture conditions. Still cultures were grown on Vogel medium N (30) containing 2% sucrose and other supplements as indicated. Cultures were prepared in 25-ml volumes in 125-ml Erlenmeyer flasks equipped with metal closures and incubated at 30 C. After incubation, the cultures were filtered, the filtrates were saved for various analyses, and the mycelium was rinsed with water and dried at 100 C overnight. Germinated conidia preparations were obtained from cultures in medium N containing 1% sucrose and other desired supplements, starting with an inoculum of 2×10^7 conidia per ml. Specific details of the production of conidia and the preparation of germinated conidia have been described elsewhere (15).

Kynureninase. Strain 74A was grown in 15 liters of medium N containing 2% sucrose and 2.0 μmoles of L-tryptophan per ml at 30 C with vigorous aeration. The inoculum was a 200-ml still culture grown in the same medium at 30 C for 48 hr, and dispersed by a brief treatment in a blendor. The culture was incubated for 48 hr, and the mycelia were harvested, washed with water, and lyophilized. The dried mycelia were pulverized in a Wiley mill equipped with a 60-mesh screen. The mycelial powder was extracted with 0.1 M sodium phosphate buffer at *p*H 8.0 (15 ml per g of powder) and stirred for 30 min at 0 C. The mixture was centrifuged at 17,000 × *g* for 30 min, and the kynureninase in the supernatant fluid was partially purified by protamine and ammonium sulfate fractionation by the method of Jacoby and Bonner (13).

NAK. A 100-ml still culture of strain Y-31881 was grown in half-strength medium N with 1% sucrose, 2 nmoles of nicotinamide per ml, and 10 μg of *i*-inositol per ml at 30 C for 48 hr and dispersed in a blendor. This was used to inoculate 16 liters of the same medium, and the culture was incubated at 30 C for 54 hr with vigorous aeration. The culture was filtered and the filtrate was concentrated to about 400 ml in a rotary evaporator. NAK was isolated from the concentrated filtrate by the method of Yanofsky and Bonner (33); the yield was 0.6 g of crystals which had an appropriate melting point (199 C, uncorrected). After acid hydrolysis and treatment with kynureninase, the molar equivalent of anthranilic acid was found, and the absorption spectra of acid and alkaline solutions matched those previously reported for NAK (33).

Assay for NAK. The assay for NAK involves the removal of the acetyl group by acid hydrolysis, neutralization with NaOH and buffer, and conversion of kynurenin to anthranilic acid by kynureninase (NAK is not affected by kynureninase). For hydrolysis, 1.0 ml of sample and 2.0 ml of 2.0 N H_2SO_4 were heated for 1.5 hr in a boiling-water bath; after cooling, 2.0 ml of 2.0 N NaOH and 5.0 ml of 0.05 potassium phosphate buffer (*p*H 8.0) were added, and the volume was adjusted to 10.0 ml with water. For kynureninase treatment, to 0.2 ml of hydrolysate was added 0.5 ml of a reaction mixture containing 50 μmoles of potassium phosphate buffer (*p*H 8.0), 4 μg of pyridoxal phosphate, 0.5 μmoles of $MgSO_4$, and 2 units of kynureninase (produces 2 μmoles of anthranilic acid in 30 min at 35 C in the same reaction mixture with saturating concentrations of kynurenin); after incubation for 60 min at 35 C, 0.5 ml 1.0 M acetate buffer (*p*H 4.0) was added, and the mixture was extracted with 5.0 ml of ethyl acetate; anthranilate was determined fluorimetrically in an Aminco-Bowman spectrophotofluorometer, with excitation at 340 nm and emission at 400 nm.

Other assays. Formamidase activity was assayed with formylanthranilic acid as a substrate by the method of Jacoby (12); formamidase specific activity is designated as the increase in optical density at 330 nm per 20 min per mg of protein at 30 C. 3-Hydroxyanthranilic acid was estimated fluorimetrically; 0.5 ml of sample was acidified with 0.5 ml of 1.0 M acetate buffer (*p*H 4.0) and extracted with 5.0 ml of ethyl acetate. The extract was analyzed with excitation at 345 nm and emission at 420 nm. Protein was measured by the method of Lowry et al. (21). Tryptophan was estimated from the indole produced after treatment of samples with tryptophanase, by the method of Frank and DeMoss (8).

RESULTS

Influence of nicotinamide on the accumulation of NAK in still culture. Strain RC-53-2A (*nic-3 kyn*) was grown in media containing various amounts of nicotinamide. Cultures were harvested at 47 and 68 hr, the cells were dried, and the filtrates were assayed for anthranilic acid, kynurenine, and NAK. The procedure described for the assay of NAK, omitting kynureninase, was used to estimate anthranilate, and the hydrolysis step (heating) was omitted for the estimate of free kynurenin. No significant amounts of anthranilate were found, and free kynurenin

amounted to no more than 2.5% of the values obtained in the complete assay for NAK.

Table 1 shows an inverse relationship between the level of nicotinamide and the accumulation of NAK. Virtually no NAK was produced at concentrations of nicotinamide above 7 nmoles/ml, which was optimal for growth in this experiment. At levels below 7 nmoles/ml, the increase in NAK from 47 to 68 hr was again inversely related to the concentration of nicotinamide, whereas the increase in cell dry weight showed a direct relationship to the level of nicotinamide. These observations suggest that NAK production continued after growth had ceased or when, presumably, nicotinamide was no longer available. Thus, it appears that nicotinamide can adversely affect the production of NAK.

Specificity of nicotinamide inhibition of the accumulation of NAK in still culture. The uniqueness of nicotinamide in affecting the accumulation of NAK was examined by testing several related compounds, isomers, conjugates, metabolites, and precursors for their effects on growth and on the accumulation of NAK. Experiment 1 of Table 2 shows that the only compounds affording a significant stimulation of growth were nicotinamide, nicotinic acid, and pyridine-3-aldehyde; the last compound might have been oxidized to nicotinic acid (during or before culture). It is notable that these compounds also effected a dramatic decrease in NAK production. In experiment 2, besides nicotinamide and nicotinic acid, nicotinuric acid and 3-hydroxy-kynurenin stimulated growth and correspondingly inhibited the accumulation of NAK. Nicotinuric acid (nicotinyl-glycine) might have been hydrolyzed to free nicotinic acid, and 3-hydroxy-kynurenin is a precursor of nicotinic acid in *Neurospora*, coming after the block in this strain. Kynurenin had no significant effect on growth, as might be expected since this strain is unable to convert kynurenin to 3-hydroxy-kynurenin, which is the next compound in the tryptophan-nicotinic pathway. The lack of effect of kynurenin on the production of NAK indicates that kynurenin neither inhibits nor stimulates the system of reactions leading to its production. Generally, then, the observed inhibitions of production of NAK appear to be specifically related to nicotinamide or to functionally equivalent compounds.

TABLE 1. *Effect of nicotinamide on the accumulation of NAK in still cultures of strain RC-53-2A (nic-3, kyn)*

Nicotinamide (nmoles/ml)	Cell dry wt (mg/flask)		NAK (nmoles/ml)	
	47 hr	68 hr	47 hr	68 hr
0	0	0	0	0
1	30	36	144	380
2	54	72	162	295
4	65	94	55	148
7	72	105	20	31
10	73	103	7	9
15	71	97	3	3

TABLE 2. *Effect of nicotinamide and related compounds on the accumulation of NAK in still cultures of strain RC-53-2A (nic-3, kyn)*

Additions to medium[a]	Amt of compound added (nmoles/ml)	Cell dry wt (mg/flask)	NAK (nmoles/ml)
Expt 1 (70 hr)			
None		54	247
Nicotinamide	10	103	2
Nicotinic acid	10	113	2
Isonicotinamide	25	54	210
Isonicotinic acid	25	50	218
Isonicotinic hydrazide[b]	25	51	218
Pyridine-3-aldehyde[b]	25	90	54
Pyridine-4-aldehyde[b]	25	52	218
6-Hydroxy-nicotinic acid[b]	25	50	225
Nikethamide[b]	25	46	181
Trigonellin[b]	25	59	239
Pyridine-3-sulfonic acid[b]	25	46	196
Picolinic acid	25	50	210
Quinolinic acid	25	52	225
Expt 2 (72 hr)			
None		63	328
Nicotinamide	10	109	2
Nicotinic acid	10	103	2
Nicotinuric acid[b]	25	87	115
L-Kynurenin[b]	25	62	301
3-Hydroxy-L-kynurenin[b]	25	105	18

[a] The basal medium was medium N containing 2% sucrose and 1.5 nmoles of nicotinamide per ml. The added compounds were obtained from commercial sources and were used without further purification or analysis.

[b] Added as filter-sterilized solutions to cooled, autoclaved basal medium.

Effect of nicotinamide on the accumulation of 3-hydroxy-anthranilic acid in still culture. The influence of nicotinamide on the steps immediately following kynurenin in the tryptophan-nicotinic acid pathway was examined with strain 4540 which accumulates 3-hydroxy-anthranilic acid (3). The accumulation of 3-hydroxy-an-

thranilic acid was determined in cultures grown on various levels of nicotinamide in the absence and presence of added kynurenin. The conversion of kynurenin to 3-hydroxy-anthranilic acid involves two reactions, a hydroxylation and the subsequent removal of the alanyl moiety of the side chain by kynureninase. In the absence of added kynurenin, the accumulation of 3-hydroxy-anthranilic acid might be expected (from the previous experiments) to be inhibited by excess nicotinamide. In the presence of kynurenin, which could circumvent the earlier steps in the pathway, the effect of nicotinamide would be exerted on endogenous kynurenin synthesis or on the conversion of exogenously supplied kynurenin to 3-hydroxy-anthranilic acid, or both.

In the absence of added kynurenin, the anticipated inverse relationship between nicotinamide concentration and the accumulation of 3-hydroxy-anthranilic acid occurred (Table 3). However, in the presence of added kynurenin, the production of 3-hydroxy-anthranilic acid markedly increased and the inhibitory effect of nicotinamide was significantly moderated. The smaller decrease in the production of 3-hydroxy-anthranilic acid in the presence of added kynurenin might be attributed to the effect of nicotinamide on the synthesis of endogenous kynurenin. These data suggest that the observed inhibitions by nicotinamide occur at a point(s) earlier than kynurenin (or NAK) in the pathway of nicotinamide biosynthesis.

TABLE 3. *Influence of nicotinamide and kynurenin on the formation of 3-hydroxyanthranilate by still cultures of strain 4540 (nic-2)*[a]

Additions to medium		Cell dry wt	3-Hydroxyanthranilic acid	
Nicotinamide (nmoles/ml)	L-Kynurenin[b] (nmoles/ml)	(mg/flask)	(nmoles/ml)	(nmoles/mg dry wt)[c]
0				
1		28	30	27
2		64	59	23
5		70	46	16
10		74	5	2
0	250			
1	250	18	29	40
2	250	42	151	90
5	250	50	179	89
10	250	51	90	44

[a] Cultures were incubated at 30 C for 80 hr.

[b] Kynurenin solutions were filter-sterilized and added to cooled, autoclaved medium.

[c] Estimated on the basis of the initial culture volume of 25 ml.

Influence of nicotinamide on the contribution of tryptophan to the accumulation of NAK by strain RC-53-2A in still culture. The experiments described above suggest that the site(s) of nicotinamide inhibition occur prior to kynurenin in the biosynthesis of nicotinic acid. Since nicotinic acid is derived from tryptophan, the possibility exists that nicotinamide might inhibit the biosynthesis of tryptophan. Previous studies (16) indicated the unlikelihood of such an inhibition, but since that work was done with other strains of *Neurospora* and under different conditions, a more direct examination of this possibility was made. The influence of nicotinamide on the production of NAK was examined in cultures grown in the absence and presence of substantial amounts of L-tryptophan. The experimental rationale was the same as in the preceding experiment; if, in the presence of tryptophan, nicotinamide does not inhibit the production of NAK, then the site of nicotinamide action precedes tryptophan, but if such an inhibition does occur then nicotinamide inhibits at a point beyond tryptophan.

The addition of tryptophan substantially increased the levels of NAK (Table 4), but even here there was a marked and progressive inhibition of the production of NAK by increasing levels of nicotinamide. The heightened production of NAK might be attributed to an increased supply of tryptophan or to the induction by tryptophan of a system converting it to NAK, or both. However, the inhibitory action of nicotinamide, with or without the addition of tryptophan, suggests that its site of action is not in the biosynthesis of tryptophan. Parenthetically, it may be noted that tryptophan inhibits the growth of

TABLE 4. *Effect of tryptophan on the inhibition by nicotinamide of the accumulation of NAK in still cultures of RC-53-2A*[a]

Additions to medium		Cell dry wt	NAK	
Nicotinamide (nmoles/ml)	L-Tryptophan (μmoles/ml)	(mg/flask)	(nmoles/ml)	(nmoles/mg dry wt)[b]
1		36	319	222
3		79	142	45
10		114	5	2
1	3	15	328	548
3	3	40	316	197
10	3	43	125	72

[a] Cultures were incubated at 30 C for 76 hr.

[b] Estimated on the basis of the initial culture volume of 25 ml.

RC-53-2A, and this is probably due to the excessive formation of kynurenin; other experiments (Table 3; *unpublished data*) have shown kynurenin to be growth-inhibiting, but the nature of this effect is not known. (Neither tryptophan nor nicotinamide reverses the inhibitory effect of kynurenin.)

The experiments described thus far strongly indicate that nicotinamide inhibits the conversion of tryptophan to NAK. In terms of the steps involved, the likely sites of nicotinamide action could be tryptophan pyrrolase (tryptophan → *N*-formylkynurenin), kynurenin formamidase (*N*-formylkynurenin → kynurenin), and kynurenin acetylation (kynurenin → NAK). The following series of experiments examines these reactions as sites of nicotinamide inhibition; they also inquire whether nicotinamide inhibits the formation or the activity (or both) of the system which converts tryptophan to NAK.

Effect of nicotinamide on the formation of NAK-synthesizing activity in germinating conidia of strain RC-53-2A. Conidia were germinated in the presence of various amounts of nicotinamide, 10-ml samples were taken for cell dry weight determinations, and the culture filtrates were analyzed for NAK. The remaining germinated conidia were washed and suspended in buffered saline (0.05 M NaCl in 0.02 M sodium phosphate buffer at *p*H 5.8). Portions of the suspensions of germinated conidia were filtered, lyophilized, powdered, and extracted with 0.05 M potassium phosphate buffer at *p*H 7.5 (25 ml per g, dry weight). The mixture was centrifuged at 27,000 × *g* for 30 min, and the supernatant fluid obtained was assayed for formamidase activity and protein content. The remaining suspensions of germinated conidia were tested for NAK-synthesizing activity, tryptophan utilization, and dry weight. The suspensions were made up to 25 ml in buffered saline containing 20 μg of cycloheximide, 2.0 μmoles of L-tryptophan, and about 5 mg (dry weight) of germinated conidia per ml. Cycloheximide was added to prevent protein synthesis. Preliminary experiments showed that, without cycloheximide, NAK-synthesizing activity increased during incubation whereas, with cycloheximide, a constant rate of NAK production occurred. The reaction mixture was incubated at 30 C in a reciprocating water-bath shaker. Samples were taken at 0, 90, and 180 min after the addition of tryptophan and filtered; the filtrates were assayed for NAK, and the cell dry weight was determined. Filtrates taken at zero time and filtrates of heated (2 to 3 min, 100 C) suspension remaining at 180 min were assayed for tryptophan; the difference in the values obtained was assumed to represent the tryptophan metabolized. No appreciable change in cell dry weight occurred. The specific activity of NAK synthesis is designated as nanomoles of NAK produced per milligram of cell dry weight per 180 min.

Maximum growth of conidia was obtained with about 5 nmoles of nicotinamide per ml (Table 5). NAK accumulation, as with still cultures, was inversely related to the concentration of nicotinamide. The formamidase activities of these cultures was the same in every case and, thus, it appears that nicotinamide does not influence the formation of formamidase. Suspensions of germinated conidia which were grown on various levels of nicotinamide also showed no significant differences in their utilization of tryptophan; the values obtained are similar to those described elsewhere (18). These results are consistent with those of Table 4 and indicate that nicotinamide does not significantly affect the uptake of tryptophan nor its metabolism to compounds other than kynurenin (22). However, the NAK-synthesizing activity of germinated conidia is inversely related to the level of nicotinamide on which they were grown.

Effect of nicotinamide on the NAK-synthe-

TABLE 5. *Effect of nicotinamide on the formation of formamidase, NAK-synthesizing activity, and tryptophan metabolism in germinated conidia of strain RC-53-2A*

Germinated conidia culture				Germinated conidia	
Nicotinamide (nmoles/ml)	Cell dry wt (mg/ml)	NAK (nmoles/ml)	Formamidase[a] (specific activity)	Tryptophan metabolized (nmoles/mg)	NAK synthesis (specific activity)
1	4.3	630	0.046	41	25
2	5.0	345	0.052	49	23
5	5.5	75	0.045	41	9
10	5.7	6	0.053	45	6
25	5.6	nil	0.044	47	2

[a] About 2 mg of protein was present in each assay.

sizing activity of germinated conidia of strain RC-53-2A. Conidia were germinated in a medium containing 1.0 nmole of nicotinamide per ml; suspensions of germinated conidia in buffered saline were tested for NAK-synthesizing activity, as described above, in the presence of various amounts of nicotinamide. Levels of nicotinamide which are well above those affording optimum growth had very little effect on preformed NAK-synthesizing activity (Table 6). In some experiments, the germinated conidia were preincubated with nicotinamide for as long as 180 min before adding tryptophan, but no significant inhibition of NAK-synthesizing activity was observed. These results, together with those in Table 5, indicate that nicotinamide may repress the formation of NAK-synthesizing activity rather than inhibit this activity once formed.

Effect of nicotinamide on the formation of kynurenin acetylating activity in germinating conidia of strain RC-53-2A. The above experiments tend to rule out formamidase or tryptophan availability as the sites of nicotinamide action. The remaining, likely possibilities are tryptophan pyrrolase and kynurenin-acetylating activities. The latter seems untenable, since free kynurenin (or *N*-formylkynurenin) does not accumulate to any significant extent. However, this possibility was examined more directly. Conidia were germinated on various levels of nicotinamide, and buffered saline suspensions of germinated conidia were tested for NAK-synthesizing activity with L-tryptophan and with L-kynurenin as substrates. Samples were taken at 0 and 180 min, cell dry weight was measured, and the reaction filtrates were assayed for kynurenin with and without prior acid hydrolysis. Where tryptophan was present, no free kynurenin was found in unhydrolyzed samples. Where kynurenin was added, the same values were obtained for unhydrolyzed samples at zero time and for hydrolyzed samples taken at 180 min, indicating no loss of total kynurenin. Unhydrolyzed samples taken at 180 min yielded lower kynurenin values, and the difference was assumed to represent acetylated kynurenin (NAK). The data in Table 7 indicate that nicotinamide does not affect the formation of kynurenin-acetylating activity in germinating conidia. Thus, of the various sites of nicotinamide action proposed earlier, all have been demonstrated as unlikely, with the exception of the first step in the tryptophan-nicotinic acid pathway, the tryptophan pyrrolase reaction.

TABLE 6. *Effect of nicotinamide on the preformed NAK-synthesizing activity of germinated conidia*

Nicotinamide (nmoles/ml)	NAK synthesis (specific activity)
0	25
5	25
25	24
100	21

TABLE 7. *Effect of nicotinamide on the acetylation of kynurenin*

Germinated conidia culture		Activity of germinated conidia	
Nicotinamide (nmoles/ml)	Cell dry wt (mg/ml)	Substrate (1.0 μmole/ml)	NAK synthesis (specific activity)
1.0	3.0	L-Tryptophan	23
		L-Kynurenin	27
2.5	5.5	L-Tryptophan	12
		L-Kynurenin	31
5.0	5.8	L-Tryptophan	1
		L-Kynurenin	28

DISCUSSION

The results of this study demonstrate that the site of nicotinamide action is the first step in the tryptophan-nicotinic acid pathway, the tryptophan pyrrolase reaction. This conclusion is based on the assumption that the synthesis of nicotinic acid proceeds from tryptophan to *N*-formylkynurenin to kynurenin, and on the elimination of all the related reactions other than the first. Tryptophan pyrrolase activity has not been demonstrated in cell-free preparations from *Neurospora*, so a clear exhibition of the inhibitory effect of nicotinamide on its activity or formation has yet to be made. However, the endogenous role of a tryptophan pyrrolase activity has been inferred from isotope studies on the conversion of tryptophan to nicotinic acid (26).

Another possible explanation for the inhibitory effect of nicotinamide on the synthesis of NAK is that nicotinamide induces or stimulates other reactions for the metabolism of tryptophan. For example, the conversion of tryptophan to indole pyruvic acid (22) could direct the flow of tryptophan away from the production of NAK. However, filtrates of still cultures and conidia germination cultures, on low and high levels of nicotinamide, showed no indole pyruvic acid or indole acetic acid when tested with Salkowski's reagent (9), which is a sensitive indicator of these compounds. This makes it more likely that the action of nicotinamide is directed toward the conversion of tryptophan to *N*-formylkynurenin.

The synthesis of NAK is specifically inhibited by nicotinamide and nicotinic acid, and only those compounds which can serve as physiolog-

ical substitutes exhibited a similar effect. However, later derivatives, such as nicotinamide adenine dinucleotide phosphate, might be the actual effectors of the inhibitions observed. This would be consonant with other work (6, 31) and with the likelihood that nicotinamide and nicotinic acid may not occur normally as free intermediates in the biosynthesis of nicotinamide adenine dinucleotide; under certain conditions a secretion of nicotinic acid has been observed (34) in *Neurospora*.

Nicotinamide apparently inhibits the formation of NAK-synthesizing activity during growth, but, once formed, this activity is no longer affected by high levels of nicotinamide. Thus it appears that nicotinamide acts as a repressor of the formation of NAK-synthesizing activity. It might be supposed that cells grown on high levels of nicotinamide might accumulate large amounts of this compound or products thereof, and these act as inhibitors of a relatively invariable NAK-synthesizing activity. This would be consistent with the in vitro action of reduced nicotinamide adenine dinucleotide phosphate as an inhibitor of tryptophan pyrrolase activity from *X. pruni* (31) and from rat liver (6). However, it has been noted earlier that in germinated conidia the rate of NAK-synthesizing activity is constant for long periods (up to 6 hr) in the presence of cycloheximide, whereas in the absence of cycloheximide the rate increases with time. This indicates that protein synthesis is involved in the formation of NAK-synthesizing activity, presumably at the expense of endogenous materials in washed, germinated conidia. The persistence of a constant rate also indicates that NAK-synthesizing activity is stable in vivo.

When nicotinamide is added to germinated conidia in reaction mixtures without cycloheximide, the increase in the rate of NAK synthesis is abolished, and the rate then observed is the same constant rate obtained in the presence of cycloheximide (J. Faris, Thesis, Reed College, 1970); the effects of cycloheximide and nicotinamide are not additive. Thus, it can be inferred that nicotinamide and cycloheximide similarly inhibit the formation of NAK-synthesizing activity. In terms of the site of action designated by this study, it appears that nicotinamide (or its derivatives) specifically represses the synthesis of tryptophan pyrrolase.

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