Metabolism of Nicotinamide and Nicotinic Acid in *Neurospora crassa*

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Nicotinic acid is outstanding among the B vitamins in that its metabolic fate has been very extensively investigated. These studies, however, have been carried out mostly in higher animals, and they have revealed that (i) *N'*-methylnicotinamide is, in general, the major end-product of the metabolism of both nicotinic acid and nicotinamide; (ii) nicotinic acid, the glycine conjugate of nicotinic acid, is also excreted in considerable proportions when nicotinic acid is administered in extraphysiological amounts; (iii) a not insignificant part of the vitamin can be accounted for as respired carbon dioxide; (iv) interconversion of the acid and amide forms of the vitamin does not generally take place on any extensive scale (Roth, Leifer, Hogness & Langham, 1948; Leifer, Roth, Hogness & Corson, 1951; Lin & Johnson, 1953; Reddi & Kodicek, 1953). The study of the metabolism of nicotinic acid and nicotinamide in *Neurospora crassa* was undertaken in this Laboratory in the course of an investigation of the comparative metabolism of the vitamin in different orders of living systems (Sundaram, Radhakrishnamurthy & Sarma, 1954; Sundaram & Sarma, 1954, 1956). After a study of the metabolism of the vitamin under growing as well as resting conditions of the mould, it has been possible to demonstrate *in vitro* the presence of an enzyme system capable of deamidating nicotinamide in cell-free extracts of the organism; further, the distribution of this enzymic activity between the fractions produced by differential centrifuging of disintegrated hyphae, as also some of the properties of the enzyme, have been investigated.

The presence of a nicotinamide deamidase was demonstrated for the first time in lactic acid bacteria (Hughes & Williamson, 1953), and two other similar systems have since been reported, both in micro-organisms (Oka, 1954; Halpern & Grossowicz, 1957).

EXPERIMENTAL

Materials

The strains of *N. crassa* used in these studies, Em5297a (wild), 39401 and 3416 (Mitchell, 1950), were maintained by fortnightly subculture on agar slants prepared from Fries's

medium (Horowitz & Beadle, 1943) supplemented with 0.2% of yeast extract (Difco) and 0.2% of malt extract (Difco), and 500 µg./100 ml. of nicotinamide with mutants 39401 and 3416. A suspension in sterile water of conidia from a 5- to 6-day-old growth from stock culture was used for all inoculations.

Methods

Metabolism of nicotinic acid and nicotinamide under growing conditions of Neurospora crassa. *N. crassa* was grown at 30° in 250 ml. Erlenmeyer flasks on the basal medium (pH 4.5) supplemented with nicotinic acid or nicotinamide (300 µg./100 ml.). At the end of the required period of growth, the mycelium was removed by filtration and the filtrate was evaporated to dryness *in vacuo* and taken up in a small volume of aq. 50–60% ethanol. After centrifuging, the clear supernatant was analysed for possible metabolites of nicotinic acid and nicotinamide by the ascending paper-chromatographic method (Sundaram *et al.* 1954).

Metabolism of nicotinamide under resting conditions of Neurospora crassa. The procedure adopted was essentially that employed by Yanofsky & Bonner (1951) for the study of the biosynthesis of nicotinic acid by the resting mycelium of *N. crassa*. *N. crassa* was grown for 96 hr. at 30° on 50 ml. lots of the basal medium supplemented with nicotinamide (100 µg./100 ml.). The mycelial pads were filtered off, washed thoroughly with sterile water, pressed dry and used in the following experiment. Three flasks, each containing 20 ml. of sterile water adjusted to pH 4.0–4.2 with dil. HCl, were incubated: one flask with nicotinamide supplement (50 µg.) and mycelium, another with nicotinamide but no mycelium and the third containing mycelium but no nicotinamide, the second and third serving as controls. The flasks were shaken continuously at 30° for 20 hr., at the end of which the mycelia were filtered off and the filtrates analysed as before for nicotinic acid, nicotinamide, nicotinic acid and *N'*-methylnicotinamide.

Deamidation of nicotinamide by cell-free extract of Neurospora crassa. Mycelial pads of *N. crassa* 39401 grown on the basal medium supplemented with nicotinamide (100 µg./100 ml.) were washed thoroughly with water, and pressed dry and chilled. The chilled mould was thoroughly ground in a mortar surrounded by a freezing mixture of ice and salt, with addition of sand (British Drug Houses Ltd., Poole, Dorset), and homogenized in ice-cold water, so that the homogenate contained the equivalent of 120 mg. of fresh mycelium/ml. The supernatant, obtained after centrifuging of the homogenate at 2000 rev./min. (1000 g) for 10 min. to sediment the sand and cell debris, was used as the enzyme source for deamidation of nicotinamide *in vitro*. The complete experimental system contained (in 4 ml.): 1 ml. of 0.2M-phosphate ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$) buffer (pH 7.0), 1 ml. of nicotinamide soln. (8 µmoles) and

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2 ml. of the enzyme preparation. In the blank, 1 ml. of water replaced the nicotinamide solution. Incubation was at 37° for 2 hr., at the end of which the ammonia and the free nicotinic acid present in the incubation mixture were determined quantitatively. In preliminary experiments, the deamidation of nicotinamide was inferred by the appearance in the incubation mixture of free nicotinic acid, which was detected by paper chromatography.

Ammonia estimation. The incubation mixture was treated with trichloroacetic acid, final concentration 2.5% (w/v), and centrifuged and the supernatant collected. The residue was washed twice with 2.5% trichloroacetic acid by centrifuging and the washings were added to the main extract. The combined supernatant, after addition of borate buffer (5% borax in 0.5N-NaOH), pH 10.0, till distinctly alkaline to phenolphthalein, was distilled *in vacuo* at 45° in a modified Parnas apparatus (Damodaran, Ramaswamy, Venkatesan, Mahadevan & Ramdass, 1946); the ammonia evolved was absorbed in 0.02N-H₂SO₄ and the solution made up to volume. A portion of the made-up solution was treated with Nessler's reagent and the colour produced measured at 490 mμ with a Lumetron photoelectric colorimeter, model 400A, Photovolt Corp., N.Y., U.S.A.

Free nicotinic acid was determined in the incubation mixture, after heating it for 15 min. in a boiling-water bath, by the microbiological procedure with *Leuconostoc mesenteroides* 9135 (Johnson, 1945), which responds specifically to free nicotinic acid.

Intracellular distribution of nicotinamide deamidase in *Neurospora crassa*. A homogenate of the mycelia of *N. crassa* was prepared as follows: the washed mycelial pads were pressed dry, then cut to small pieces, and chilled and, after preliminary grinding to a paste (without addition of sand) in a chilled mortar, suspended in isotonic sucrose (0.25M) and immediately passed through a Teflon-pestle homogenizer assembly (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) at 0°. The fractionation of the homogenate into particulate components corresponding to the nuclei, mitochondria and submicroscopic particles of animal tissues and the soluble supernatant was by the low-temperature differential-centrifuging method of Schneider (1948) on an International refrigerated centrifuge (model PR 2). The various fractions thus obtained and a part of the unfractionated homogenate were made up in isotonic sucrose to give in each case a concentration equivalent to approximately 8% (w/v) of fresh *Neurospora* mycelium. The enzyme activities in the various fractions as well as in the whole homogenate were determined by estimating the

free nicotinic acid liberated from nicotinamide during an incubation period of 2 hr. as previously described.

Routine estimation of enzyme activity. For routine estimation of enzyme activity, as in the study of the properties of the enzyme and in the survey of the distribution of the enzyme in different strains of *N. crassa*, a 10% (w/v) homogenate of the fresh mycelium was prepared in the Teflon-pestle homogenizer as described above, and the supernatant, after centrifuging at 0° for 1 hr. at 18 000 g, served as the enzyme source. The free nicotinic acid released in a 2 ml. system [0.5 ml. of buffer, pH 7.5 (40 ml. of 0.8N-KH₂PO₄ mixed with 17 ml. of 0.8N-NaOH), 0.5 ml. of nicotinamide soln. (4 μmoles) and 1 ml. of enzyme extract] during an incubation period of 2 hr. at 37° was determined microbiologically.

Nitrogen in enzyme extract. Nitrogen in the enzyme extracts was determined by the micro-Kjeldahl method, involving nesslerization of the H₂SO₄-H₂O₂ digest (Koch & McMeekin, 1924).

RESULTS

In the experiments on the metabolism of nicotinic acid and nicotinamide under growing conditions of *N. crassa*, the major metabolite present in the culture medium, as revealed by paper chromatography, was free nicotinic acid, irrespective of whether the medium had been originally supplemented with nicotinic acid or nicotinamide. Neither *N'*-methylnicotinamide nor nicotinuric acid could be detected in any of the culture media. Thus the main feature of the metabolism of the vitamin in the mould is the deamidation of nicotinamide. This capacity of *N. crassa* to convert nicotinamide into nicotinic acid observed under growing conditions was exhibited also under resting conditions of the organism. Here again no other metabolites such as *N'*-methylnicotinamide and nicotinuric acid could be detected in the medium. The data recorded in Table 1 constitute a demonstration of the presence of an enzyme system in *N. crassa* which can convert the amide form of the vitamin into the acid form *in vitro*, and reveal that during this process nicotinic acid and ammonia arise in stoichiometric proportions, the ratio of ammonia liberated to nicotinic acid released being

Table 1. *Deamidation of nicotinamide by cell-free extract of Neurospora crassa*

At the end of incubation, an amount of nicotinamide was added to the blank equal to that added to the test system before incubation. For experimental details see text.

Time of growth of <i>N. crassa</i> (hr.)	Experimental system	Ammonia present in 100 ml. of incubation mixture (μmoles)	Ammonia liberated by deamidation of nicotinamide (μmoles)	Nicotinic acid present in 100 ml. of incubation mixture (μmoles)	Nicotinic acid released by deamidation of nicotinamide (μmoles)	Ammonia liberated Nicotinic acid released
120	Blank	44.0	21.2	6.5	19.5	1.09
	Test	65.2		26.0		
192	Blank	40.2	17.6	Negligible	14.9	1.18
	Test	57.8		14.9		

Table 2. *Distribution of nicotinamide deamidase activity in the subcellular components of Neurospora crassa*

Incubation system: 1 ml. of 0.2 M-phosphate buffer, pH 7.0, 1 ml. of nicotinamide (8 μ moles) (1 ml. of water in blank), 2 ml. of cell preparation. Incubation was at 37° for 2 hr. Fractions I, II, and III correspond respectively to the nuclei, mitochondria and submicroscopic particles of animal tissues (Schneider, 1948).

Cell fraction	Details of separation	Nicotinic acid released by 2 ml. of cell fraction (μ m-moles)	Percentage of total activity in fraction	Nitrogen in 2 ml. of cell fraction (mg.)	Nicotinic acid released/mg. of N of fraction (μ m-moles)
Unfractionated homogenate	—	610	100.0	3.85	158
Fraction I	Precipitate on centrifuging at 600 g for 10 min.	94	15.4	1.5	63
Fraction II	Precipitate on centrifuging at 8500 g for 10 min.	0	0.0	—	—
Fraction III	Precipitate on centrifuging at 18 000 g for 1 hr.	0	0.0	—	—
Fraction IV	Supernatant after centrifuging at 18 000 g for 1 hr.	545	89.3	1.34	407

Table 3. *Nicotinamide deamidase in different strains of Neurospora crassa*

The different strains were grown for 72 hr. on basal medium with the appropriate supplement as indicated. Enzyme activity was determined in 1 ml. of supernatant from a 10% homogenate of the mycelium. The incubation system comprised 0.5 ml. of NaOH-KH₂PO₄ buffer, pH 7.5, 0.5 ml. of nicotinamide soln. (4 μ moles), 1 ml. of enzyme extract. Incubation was at 37° for 2 hr.

<i>N. crassa</i> strain	Supplement to growth medium (500 μ g./100 ml.)	Nicotinic acid released in 2 ml. of system (μ m-moles)
39401	Nicotinamide	437
Em 5297a (wild)	Nil	595
3416	Nicotinamide	307
39401	Nicotinic acid	545

Table 4. *Stability of nicotinamide deamidase of Neurospora crassa at different temperatures*

1 ml. of supernatant from 10% *N. crassa* homogenate was held at the appropriate temperature for the indicated period of time, at the end of which the enzyme activity was determined as described in Table 3.

Temperature	Time	Destruction of enzyme activity (%)
-20°	72 hr.	28
5	24 hr.	29
5	72 hr.	52
75	6 min.	100
75	3 min.	100
100	5 min.	100
100	2 min.	100

very nearly 1:1 on a molar basis. Fractionation of the mycelial homogenate into subcellular components revealed that the nicotinamide deamidase activity is localized almost exclusively in the

Fig. 1. pH optimum of *Neurospora crassa* nicotinamide deamidase. The enzyme activity in 1 ml. of supernatant from a 10% homogenate of the mycelium was determined as in Table 3 at different pH values (NaOH-KH₂PO₄ buffer).

soluble portion of the cytoplasm (Table 2). The small amount of deamidation associated with the fraction sedimenting at 600 g was probably due to the presence therein of a small percentage of intact cells.

The results of further studies on the nicotinamide deamidase of *N. crassa* have been recorded in Tables 3-6 and in Figs. 1 and 2. Evidence for the constitutive nature of the nicotinamide de-

amidase of *N. crassa* was obtained from experiments which showed that the enzyme was present even in the wild strain grown in the absence of nicotinamide or nicotinic acid as well as in strain 39401 grown in the presence of nicotinic acid (Table 3); the enzyme content of the wild strain, in fact, was significantly higher than that of the mutant strains, even though the latter had been grown in the presence of relatively high concentrations of nicotinamide. Data presented in Table 4 indicate that the enzyme is relatively unstable, about 30 % of its activity being lost in 24 hr. at 5°. This instability made purification of the enzyme difficult. An acetone-dried powder prepared from the supernatant of a *N. crassa* homogenate was

Table 5. Nicotinamide deamidase activity of *Neurospora crassa* at various incubation temperatures

Enzyme activity in 1 ml. of supernatant from 10 % homogenate was determined as described in Table 3 at the temperature indicated.

Temp. of incubation	Nicotinic acid released in 2 ml. of system (μ m-moles)
25°	195
32	312
37	454
45	490
52	467
60	316

Fig. 2. Effect of substrate concentration on *Neurospora crassa* nicotinamide deamidase activity. The activity in 1 ml. of supernatant from a 10 % homogenate of *N. crassa* mycelium at varying substrate concentrations was determined as in Table 3.

found to retain only 40 % of the enzyme activity. The enzyme has a rather broad range of optimum pH (8.5–9.0; Fig. 1). It exhibits maximum activity at about 45° during an incubation period of 2 hr. (Table 5); in view of the rapid inactivation of the enzyme at higher temperatures (Table 4), this result might represent the balance between the activity of the enzyme and its thermal inactivation, and it is possible that the temperature optimum will be higher than 45° if the incubation period is shortened. In Fig. 2 is recorded the activity–substrate concentration relationship of the nicotinamide deamidase of *N. crassa*. In studies on the time course of the enzyme reaction, the deamidation under the conditions of Table 3 was linear up to 100 min.; under the same conditions, the activity increased linearly with enzyme concentration up to 1 ml. of the enzyme extract.

The effects of various compounds and enzyme inhibitors on the nicotinamide deamidase of *N. crassa* are shown in Table 6. The heavy-metal ions Cu^{2+} and Hg^{2+} almost completely inhibited the enzyme; of the rest, only the metal-chelating agents, $\alpha\alpha'$ -dipyridyl, 8-hydroxyquinoline and ethylenediaminetetra-acetate, inhibited the activity to a marked extent, indicating a requirement for metal activator(s) for enzyme action. Although *p*-chloromercuribenzoate lowered the enzyme activity by about 20 %, the inability of cysteine to reverse this inhibition and the failure of *o*-iodosobenzoate to inhibit the enzyme do not point to any definite function for –SH grouping in the enzyme action.

Table 6. Effect of various compounds and inhibitors on nicotinamide deamidase of *Neurospora crassa*

The enzyme activity in 1 ml. of supernatant from a 10 % homogenate of *N. crassa* was determined in the presence of each of the compounds at the concentration indicated, as described in Table 3.

Compound or inhibitor	Concentration (mm)	Percentage of activity of control
None (control)	—	100.0
Hg^{2+} ions*	1	5.2
Cu^{2+} ions*	1	10.2
Sodium azide	1	95.9
Isoniazid	1	103.5
Isonicotinic acid	1	94.2
Hydrazine	1	92.6
Sodium cyanide	1	95.2
<i>o</i> -Iodosobenzoate	0.01	99.6
<i>p</i> -Chloromercuribenzoate	0.01	80.5
<i>p</i> -Chloromercuribenzoate + mm-cysteine	0.01	88.3
8-Hydroxyquinoline	10	11.3
$\alpha\alpha'$ -Dipyridyl	10	14.7
$\alpha\alpha'$ -Dipyridyl	2	64.3
Ethylenediaminetetra-acetate	10	71.1
Ethylenediaminetetra-acetate	20	61.6

* Incubation mixture was buffered with 2-amino-2-hydroxymethylpropane-1:3-diol.

DISCUSSION

It has been reported earlier (Sundaram *et al.* 1954) that the insect larva *Corcyra cephalonica* St. does not methylate exogenous nicotinamide, but deamidates it to free nicotinic acid, which is thus the principal excretory metabolite of the vitamin in this invertebrate organism. The present investigation demonstrates that *N. crassa* disposes of the vitamin as nicotinic acid and further establishes the presence of the enzyme, nicotinamide deamidase, in the mould. A similar enzyme system has been observed in the *Corcyra* larva (results to be published). *N. crassa* thus appears to resemble the insect larva rather than higher animals like the rat in respect of the metabolism of nicotinic acid and nicotinamide.

In contrast with the methylation of the vitamin and its conjugation with glycine, the deamidation of nicotinamide does not appear to have been recognized as a major metabolic pathway. Investigations which are being carried out in this Laboratory have shown that the tissues of at least two avian organisms, namely the chick and the pigeon, contain nicotinamide deamidase in even greater abundance than either *N. crassa* or *Corcyra*. This would indicate that the deamidation of nicotinamide is not a metabolic reaction confined exclusively to the lower organisms. Relevant in this context is also the fact that birds excrete free nicotinic acid (Komori & Sendju, 1926; Chang & Johnson, 1957), that the pigeon liver is deficient in the enzyme nicotinamide methylkinase, which methylates nicotinamide (Cantoni, 1951), and that the chick does not excrete *N'*-methylnicotinamide as shown by Chang & Johnson (1957) with ¹⁴C-labelled nicotinamide. This, taken in conjunction with the results that have been obtained for *N. crassa* and *Corcyra*, suggests that methylation and deamidation of nicotinamide are alternative pathways in the metabolism of the vitamin.

The inability of *N. crassa* to conjugate nicotinic acid with glycine is also striking. It is generally recognized that nicotinuric acid formation is in the nature of a detoxication. Thus the higher animals, such as the rat and man, excrete nicotinuric acid extensively when nicotinic acid is administered in relatively massive amounts (Lin & Johnson, 1953; Reddi & Kodicek, 1953; Sundaram & Sarma, 1956). *Corcyra cephalonica* also excretes nicotinuric acid, to a smaller but significant extent (Sundaram, 1955). It has also been observed in the course of the present studies that nicotinuric acid cannot replace nicotinic acid or nicotinamide for the growth of *N. crassa* 39401, showing that neither does the organism possess the enzyme system capable of splitting the glycine conjugate of the vitamin.

SUMMARY

1. The metabolism of nicotinamide and nicotinic acid has been investigated in *Neurospora crassa*. It has been found that the mould neither methylates the vitamin nor conjugates it with glycine and that the predominant end-product of the vitamin metabolism is free nicotinic acid, even nicotinamide being deamidated.

2. The deamidation of nicotinamide occurs also with *N. crassa* mycelium under resting conditions.

3. The presence of an enzyme capable of releasing free nicotinic acid and ammonia from nicotinamide in stoichiometric proportions has been demonstrated in cell-free extracts of *N. crassa*.

4. Studies on the fractionation of *N. crassa* into its subcellular components have revealed that the nicotinamide-deamidating activity is localized in the soluble portion of the cytoplasm.

5. The enzyme is constitutive in nature; it is present in the wild as well as mutant strains of the mould, and its formation is independent of the presence of nicotinamide or nicotinic acid in the culture medium.

6. Some properties of the enzyme have been investigated.

7. The comparative metabolism of the vitamin in different orders of living systems and the significance of the deamidation of nicotinamide in this context have been discussed.

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Calcium and Magnesium Metabolism in Calves

2. EFFECT OF DIETARY VITAMIN D AND ULTRAVIOLET IRRADIATION ON MILK-FED CALVES*

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It has previously been reported (Smith, 1957) that calves given a diet consisting almost exclusively of milk with no supplementary vitamin D showed, as they got older, a progressive decrease in the percentage of dietary calcium and magnesium utilized. The addition of sufficient vitamin D to the diet maintained calcium retention at a high level and prevented or cured the hypocalcaemia which frequently also occurred, but it appeared that abnormally large amounts of the vitamin were sometimes required to achieve this. Parr (1957) has also reported hypocalcaemia in milk-fed calves receiving vitamin D in amounts usually considered to be adequate. Part of the object of the present work therefore was to attempt to obtain general confirmation of these results and to examine further the effect of vitamin D levels on calcium utilization in milk-fed calves.

Our previous results (Smith, 1957) suggested that the fall which occurred in magnesium utilization with age and the accompanying hypomagnesaemia were not appreciably influenced by feeding with high levels of vitamin D. Other workers (e.g. Huffman, Conley, Lightfoot & Duncan, 1941; Duncan, Huffman & Robinson, 1935; Parr, 1957) have also demonstrated that milk-fed calves become hypomagnesaemic, usually before 3–4 months of age, and that vitamin D added to the diet does not prevent this. Herman (1936), however, kept eight calves for about 1 year or more on a milk diet with and without vitamin D supplements, and yet reported that no hypomagnesaemia occurred in any of his animals. The only manner in which his treatment appeared to differ appreciably from that of other workers was in the fact that his

calves were kept in the sunlight as much as possible. This suggested that ultraviolet-light radiation might produce an effect not shown by the oral administration of vitamin D. A further part of the present work was undertaken in order to examine this possibility.

EXPERIMENTAL

Nine bull calves were used. One was an Angus × Ayrshire crossbred (7A), another was an Angus × Shorthorn/Friesian crossbred (11A) and the remainder were Friesians. Apart from the differences described below they received the same general treatment as did the calves used previously (Smith, 1957). They were given 4.40 l. of milk/day up to 10 weeks of age, 6.15 l. of milk/day from 10 to 20 weeks and 8.80 l. of milk/day from 20 weeks. The milk was sampled every day and magnesium and calcium were determined on 4-day batches. The mean magnesium and calcium contents of the batches were 12.5 and 121 mg./100 ml. respectively, measured over the whole experimental period. The standard deviations for individual 4-day batch determinations were ± 0.6 mg./100 ml. for magnesium and ± 5 mg./100 ml. for calcium. There were no marked changes in milk magnesium from one part of the experimental period to another such as occurred in 1954–55 (Smith, 1957), and it is not therefore considered necessary to give these milk analyses in detail. The calves were given a mineral supplement containing iron, copper and manganese as in the previous work (Smith, 1957). Details of the supplementary vitamin D added to the diet and the ages at which some of the calves were irradiated with u.v. light are shown in Table 1. In addition to the vitamin D supplements, the calves received whatever naturally occurring vitamin D activity was present in their milk diet. No vitamin D assay was carried out on the milk but it is probably valid to use the figures obtained by Henry & Kon (1942). These workers studied milk obtained from the same farm as that from which our milk was obtained. They

* Part 1: Smith (1957).