Biosynthesis of Trigonelline*

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Trigonelline, N-methyl nicotinic acid, was first noted in the seeds of Trigonella foenum-graecum (the fenugreek) (1) and has since been found in the seeds and tubers of many species (2, 3) as well as in leaves of one species of pea (4). Although one may assume that trigonelline arises metabolically from nicotinic acid, definitive evidence has been lacking. In the course of several attempts to demonstrate formation of nicotinic acid from tryptophan by higher plants, administration of C\textsuperscript{14}-labeled tryptophan (5, 6) or 3-hydroxyanthranilic acid-7-C\textsuperscript{14} (7) did not result in the appearance of either labeled nicotinic acid or trigonelline. In the studies to be reported below, it has been demonstrated that trigonelline synthesis in the green pea, Pisum sativum, proceeds in a manner analogous to the formation of N-methyl nicotinamide in rat liver (8), i.e. methylation is accomplished by reaction between nicotinic acid and S-adenosyl methionine.

EXPERIMENTAL PROCEDURE

Materials and Methods

Nicotinic acid-7-C\textsuperscript{14} with specific activities of 3.09 and 5.88 mc per m mole, and nicotinamide-7-C\textsuperscript{14}, specific activity 1.4 mc per m mole, were obtained from the New England Nuclear Corporation. S-Adenosyl methionine was prepared as described by Sarett et al. (10). Trigonelline was prepared from nicotinic acid and methyl iodide as described by Phan (5, 6) or 3-hydroxyanthranilic acid-7-C\textsuperscript{14} (7) did not result in the appearance of either labeled nicotinic acid or trigonelline.

The purity of the preparation was determined by estimation of amino nitrogen and of absorbancy at 260 m\mu. Trigonelline was prepared from nicotinic acid and methyl iodide as described by Sarett et al. (10).

Paper chromatography was performed by the descending technique with the following solvent systems:

1. The upper phase of n-butanol-glacial acetic acid-water (250:60:250).
2. 70\% Ethanol + 30\% ammonium acetate, adjusted to pH 5 with HCl.
3. n-Butanol saturated with 15\% ammonium hydroxide.
4. Isopropanol 80\% made 2 n with respect to HCl.
5. 95\% Ethanol + concentrated ammonium hydroxide (95:5).
6. The upper phase of butanol-acetone-water (45:5:50).

Of these solvents, System I effects separation of nicotinic acid from trigonelline, 2 separates nicotinic acid, DPN, and trigonelline, 3 separates nicotinamide from nicotinic acid and both 5 and 6 separate trigonelline from N-methyl nicotinamide.

Radioactivity on paper chromatograms was assayed either with a Forro strip counter with an Esterline-Angus recorder, or sections of the paper were cut and examined in a Packard Tri-Carb liquid scintillation counter, or the spots were treated with water, the eluates being dried in planchets and radioactivity assayed with a Tracerlab, Inc., gas flow counter.

Preparation of Pea Extracts—Seeds of P. sativum were washed repeatedly with distilled water followed by two successive washings with 0.2\% HgCl\textsubscript{2} in 50\% ethanol and then two washes with sterile distilled water. Germination was accomplished by placing the peas on filter paper over a pad of vermiculite. The filter paper was maintained constantly soaked with sterile tap water. After 6 days of germination in the dark, the seedlings were weighed and homogenized with 2 volumes of 0.01 m Tris, pH 7.4, for 1 minute in a Waring Blender. The homogenate was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 18,000 r.p.m. for 20 minutes in a Lourdes model AB high-speed centrifuge. The entire procedure was conducted in the cold room at 3\°. The supernatant fluid so obtained was used as a source of enzymes. Better extraction of active enzymes was achieved with 0.01 m Tris at pH 7.4 than with phosphate or carbonate buffers at the same or at 0.1 m concentration and at either pH 7.4 or 8.6.

Enzymic Assays—Composition of individual reaction mixtures will be indicated separately. After incubation, tubes were cooled and 0.3 ml of 5\% trichloroacetic acid per ml of incubation mixture was added. The precipitated protein was removed by centrifugation and 25 \mu of the neutralized supernatant fluid and 10 \mu g each of trigonelline and nicotinic acid were spotted on Whatman No. 1 filter paper. Chromatograms were prepared by the descending technique with Solvent 1 for 16 to 18 hours and then dried in a current of warm air. Spots were marked under ultraviolet light and radioactivity was assayed as previously described.

RESULTS

Table 1 indicates that extracts prepared from germinating peas can effect synthesis of trigonelline from nicotinic acid and methionine in the presence of ATP. Thus, incubation of 100 \mu moles of nicotinic acid-7-C\textsuperscript{14} with 5 \mu moles of methionine resulted in formation of 2.16 m\mu moles of a radioactive compound which appeared to be trigonelline. In the absence of ATP, absolutely no synthesis was observed.

Methylation of nicotinamide by methionine in the presence of ATP with rat liver enzymes is markedly enhanced by the addition of sulfhydryl compounds such as glutathione. The glutathione appears to stimulate formation of S-adenosyl methionine from ATP and methionine, rather than the methylation of nicotinamide (11). In contrast, addition of glutathione to the pea extracts in the present study in amounts of 10 \mu moles per ml of incubation medium was without effect on the rate of trigonelline synthesis. However, the absolute requirement for ATP for trigonelline synthesis strongly suggested the possibility that
incubated at 37° for 20 hours. Where so indicated, 100 mmoles of Mg++, 20 pmoles of potassium phosphate, pH 7.4, 20 pmoles of O2, 2982 Biosynthesis of Trigonelline Vol. 235, No. 10

appear that in pea extracts, as in liver, S-adenosyl methionine and ATP were used. Further addition of ATP and aliquot was used for chromatography; radioactivity was determined with the Tri-Carb liquid scintillation counter.

omitted, Tris of the same concentration was substituted. A 25-~1 aliquot was used for chromatography; radioactivity was determined with the Tri-Carb liquid scintillation counter.

The resulting solution, free from trichloroacetic acid, was chromatographed in Solvent 1. The newly formed radioactive material was chromatographed in Solvent 1. The radioactive compound so obtained was identified as trigonelline by the following criteria:

1. An aliquot of the solution was spotted together with synthetic trigonelline, and chromatograms were obtained with each of the 6 solvents described above. In all cases, radioactivity moved with the trigonelline. Fig. 1 shows separation of the enzymatically synthesized trigonelline from N-methyl nicotinamide after 48 hours of paper chromatography with Solvent System 5. The radioactive spot so obtained was exposed to ethyl methyl ketone-ammonia mixture and then examined under ultraviolet light. In contrast with quaternary pyridinium compounds containing a CONH-R side chain, such as N-methyl nicotinamide, the enzymatically synthesized radioactive material failed to quench the irradiating light (12). Finally, the biosynthetic material served as a source of nicotinic acid for the growth of Torula cerevisiae, ATCC-2512, when examined by the technique of Johnson et al. (13), whereas N-methyl nicotinamide was not so utilized, in conformity with previous findings. Thus, chromatographically, chemically, and as a growth factor for yeast, the biosynthetic material appears to be trigonelline rather than N-methyl nicotinamide.

Properties of Enzyme—In studying the methylating enzyme from pea extracts, a fresh supernatant solution was prepared each day. A wide variation in enzyme activity was noticed although the protein content of the extracts did not differ appreciably. K_m for nicotinic acid with this enzyme, as determined from the Lineweaver-Burk plot shown in Fig. 2, was found to be 4.0 X 10^-4 M. Optimal activity was achieved between pH 6.0 and 7.0.

As indicated above, there is no evidence that these pea extracts synthesize N-methyl nicotinamide. When nicotinamide replaced nicotinic acid in the incubation medium, the resulting methylated compound was again found to be trigonelline and no traces of N-methyl nicotinamide were detected. As shown in Fig. 3, the extracts used nicotinamide for trigonelline synthesis, initially at a decidedly slower rate, but after approximately 2 hours the activity curves were almost parallel. This finding suggested the presence in the pea extract of a nicotinamidase which hydrolyzes nicotinamide to nicotinic acid and ammonia. As shown in Table II, the pea extracts exhibited vigorous deamidase activity which does not require ATP or phosphate for maximal activity and is not inhibited by the addition of ethylenediaminetetraacetate to the medium. The specificity of the deamidase was not investigated other than to establish its inactivity with respect to hydrolysis of N-methyl nicotinamide. When pea extract was incubated with 400 mmoles of N-methyl

\[ \text{Addition} \quad \text{Omission} \quad \text{Trigonelline synthesized} \]

\[ \begin{array}{ccc}
\text{S-Adenosyl methionine} & \text{ATP} & 2.16 \\
\text{S-Adenosyl methionine} & \text{ATP, methionine} & 32.4 \\
\text{S-Adenosyl methionine} & \text{ATP, methionine, Mg++, phosphate} & 31.6 \\
\end{array} \]

\( S\)-adenosyl methionine might be an intermediate. As shown in Table I, when \( S\)-adenosyl methionine was substituted for the combination of methionine and ATP, trigonelline synthesis occurred more than 10 times as rapidly as that observed when methionine and ATP were used. Further addition of ATP and methionine did not augment the rate of trigonelline synthesis observed with \( S\)-adenosyl methionine alone. Thus, it would appear that in pea extracts, as in liver, \( S\)-adenosyl methionine serves as an active donor in methylating processes.

Identification of Trigonelline—To establish the identity of the material thought to be trigonelline in the experiments described above, a mixture containing 4 times as much of each of the components as in the standard experiment was incubated in the same way. The entire protein-free filtrate was shaken 3 times with 3 volumes of ether and the residual ether removed under vacuum. The resulting solution, tree from trichloroacetic acid, was chromatographed in Solvent 1. The newly formed radioactive material, with an RF of 0.20, was located, the section of paper cut out, and eluted with water by descending chromatography. This procedure effectively separated a known sample of trigonelline from N-methyl nicotinamide (II) (for details, see text).

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tracts. The incubation mixture contained 0.4 ml of pea extract, 100 μmoles of nicotinamide-7-C₁⁴ (●), or nicotinamide-7-C₁⁴ (□). The incubation mixture was chromatographed in Solvent 3. The radioactive spots were eluted into planchets, which were dried, and assayed in a gas flow counter.

Fig. 3. Synthesis of trigonelline from nicotinamide by pea extracts. The incubation mixture contained 0.4 ml of pea extract, 100 μmoles of nicotinamide-7-C₁⁴ (●) or nicotinamide-7-C₁⁴ (□), 100 μmoles of S-adenosyl methionine, 25 μmoles of Tris acetate buffer at pH 7.4 and 12.5 μg of anreomycin in a total volume of 1.0 ml. Trigonelline synthesis was assayed as described for the experiments in Table I.

TABLE II
Additions and omissions Nicotinic acid formed μmoles

| Complete system | 100.00 |
| — ATP | 117.00 |
| — ATP — phosphate | 108.00 |
| — ATP — phosphate — Mg ++ + EDTA* 10 μmoles | 108.02 |

* Ethylenediaminetetraacetate.

nicotinamide, and the incubation mixture was chromatographed in Solvent System 3, no trigonelline could be detected on the chromatogram and the N-methyl nicotinamide was recovered quantitatively.

DISCUSSION

The results presented above strongly suggest that pea extract catalyzes the formation of S-adenosyl methionine from methionine and ATP, and demonstrate that this material can then be utilized for the methylation of nicotinic acid with formation of trigonelline. The substrate specificity of the methylating enzyme, for which the term nicotinic acid methylase is here proposed, has not been established except to indicate that it is incapable of the methylation of nicotinamide, in contrast to the enzyme in mammalian liver. The plant enzyme for S-adenosyl methionine synthesis differs from that in liver in that there is no requirement for addition of sulfhydryl compounds to the medium whereas the animal enzyme is markedly sulfhydryl-dependent. Definitive evidence of the formation of S-adenosyl methionine in plants has been provided by the isolation of this compound from extracts of barley seedlings after incubation with methionine and ATP by Mudd (14), who has also shown that S-adenosyl methionine is the methyl donor in the enzymic formation of a series of alkaloids.

It is difficult to reconcile the complete failure of N-methyl nicotinamide synthesis in pea extracts with the report of Stul'nikova (15) describing synthesis of N-methyl nicotinamide from methionine and nicotinamide in bean, wheat, and corn seedlings. Whether this reflects a species difference or differences in technique remains to be ascertained. The nicotinamide deamidase activity of pea extracts is striking in that it is considerably greater, on a molar basis, than the methylating system. The source of nicotinamide which might serve as substrate for this activity is unknown. Neither whole homogenates nor extracts of germinated peas showed any DPNase activity, and unpublished studies performed earlier failed to reveal any pathway for direct synthesis of nicotinamide from nicotinic acid, ATP, and a series of potential ammonia donors.

It is presumed that trigonelline, normally present in seeds, serves as a storage of nicotinic acid for utilization in DPN biosynthesis. The mechanism of this process is currently under investigation.

SUMMARY

Extracts of seedlings of Pisum sativum catalyze synthesis of S-adenosyl methionine from adenosine triphosphate and methionine, and synthesis of trigonelline from nicotinic acid and S-adenosylmethionine. The designation nicotinic acid methylase is suggested for the enzyme responsible for the latter reaction. Some aspects of the specificity and kinetic properties of the enzyme are presented.

REFERENCES


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1 L. Lack and P. Handler, unpublished data.
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