A Quantitative Examination of Nicotinamidase Activity in Various Tissues of Ricinus Communis L.[1]

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Hydrolysis of nicotinamide in a biological system was first reported by Hughes and Williamson (1952). Since then, nicotinamidase activity has been reported in mammals (Petrack *et al.*, 1963a, b), birds (Curti and Porcellati, 1963), microorganisms, cell-free extracts of micororganisms (Pichappa and Shanmugasundaram, 1952) and plants (Sarma, Rajalakshmi, and Sarma, 1964).

Petrack *et al.* (1963a, b) showed that high concentrations of nicotinic acid inhibit NAD synthesis *in vivo* from nicotinamide or nicotinic acid. Their data suggest that nicotinamide can serve as a generating system for maintainance of non-inhibitory levels of nicotinic acid. Joshi and Handler (1962) have purified a nicotinamidase from *Torula cremoris* and found that its biosynthesis was depressed by NAD in the medium.

Waller and Henderson (1961) have shown that nicotinamide 7-^{14}C,^{15}N can serve as a precursor to the nitrile group of ricinine, the alkaloid produced by *Ricinus communis* L. The ^{14}C:^{15}N ratio was 1:1 in the CN group of ricinine 3-4 days after administration of the nicotinamide 7-^{14}C,^{15}N but at the end of one week about 25% of the ^{15}N appeared in the pyridine ring (Waller, 1961). The logical way for this to occur would be by hydrolysis to yield nicotinic acid and $^{15}NH_2$ which could go into the amino nitrogen pool of the plant. This report establishes the occurrence of a nicotinamidase in the castor plant. Furthermore it provides the first comparative study of this enzyme in a biological system.

Experimental

Materials.—Nicotinamide-7-^{14}C was obtained from Calbiochem Corporation and ATP from Pabst Laboratories. The acetone powders used as the enzyme source were prepared from *Ricinus communis* L., Hale variety, grown at the University of California Agricultural Experiment Station, Davis, California. The powders were stored in sealed jars at 4C for about one year prior to use.

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Assay Procedure.—The homogenates were prepared as follows: 25 ml of 0.1 M tris buffer pH 7.6 was added to 1 g of acetone powder and the mixture thoroughly homogenized with a mortar and pestle. The suspension was gently stirred at 4C with a magnetic stirrer for one hour and filtered through four layers of cheesecloth. The pH of the homogenate was checked throughout the extraction procedure and adjusted to pH 7.6 when necessary. The crude homogenate was centrifuged at 27,000*g* for 20 min. The supernatant solution was used for the enzyme assay. The protein concentration of the homogenates was determined by the method of Lowry *et al.* (1951).

The standard reaction mixture contained 2 ml of cell-free extract, 0.4 ml of 0.5 M tris buffer pH 7.6, 1 μmole of nicotinamide-7-^{14}C having a specific activity of 0.46 μc/μmole and deionized water to yield a final volume of 4.0 ml. The reaction was initiated by addition of the radioactive nicotinamide. The reaction mixtures were incubated at 37C for two hours on a mechanical shaker. The enzymic action was stopped by heating the reaction flasks on a boiling water bath for 3 min. After removal of the denatured protein, 0.1 ml of the reaction mixture was subjected to descending paper chromatography on Whatman No. 1 in n-butanol saturated with 15% ammonium hydroxide (Joshi and Handler, 1962). The decrease in concentration of nicotinamide was determined by triangulation of the peaks obtained from scans of the chromatography strips with a Nuclear Chicago Model C-100-B 4π Scanner in conjunction with a Nuclear Chicago Model R-1000-A Recorder. The specific activity of the enzyme preparations is defined as decrease in area of the nicotinamide-7-^{14}C peak per hour per milligram of protein per 0.1 ml assay volume.

Table 1. *QUANTITATIVE DATA ON NICOTINAMIDASE ACTIVITY IN CELL FREE EXTRACTS OF VARIOUS TISSUES OF THE CASTOR BEAN PLANT*

Tissue	$-\Delta$ Area/hour (cm^2)	mg protein/ assay	Specific Activity*	Relative Enzyme Activity**
12 day-old seedlings	17.0	0.173	98.3	1.000
Small stems	4.9	0.093	52.7	0.535
Large seeds	3.2	0.088	36.4	0.370
Male flowers	2.8	0.109	25.7	0.261
Large leaves	2.6	0.109	23.9	0.243
Small leaves	4.1	0.223	18.4	0.187
Immature seeds	2.8	0.208	13.5	0.137
Flowers and buds	2.5	0.206	12.1	0.123
Female flowers	2.2	0.215	10.2	0.104

* Specific activity as defined in the text under assay procedure.

** Enzyme activity relative to 12 day-old seedlings.

Table 2. *EFFECT OF ATP AND Mg^{++} ON ENZYMATIC ACTIVITY OF CELL FREE HOMOGENATES OF LARGE LEAVES*

Soluble Extract 27,000 x *g* Supernatant	Area of NAm Peak	% Stimulation
No cofactors	22.2 cm^2	—
+ Mg^{++} (5 μ Mole)	18.4	17.1
+ ATP (40 μ Mole)	20.0	9.9
+ ATP (40 μ Mole) and Mg^{++} (5 μ Mole)	11.0	52.5

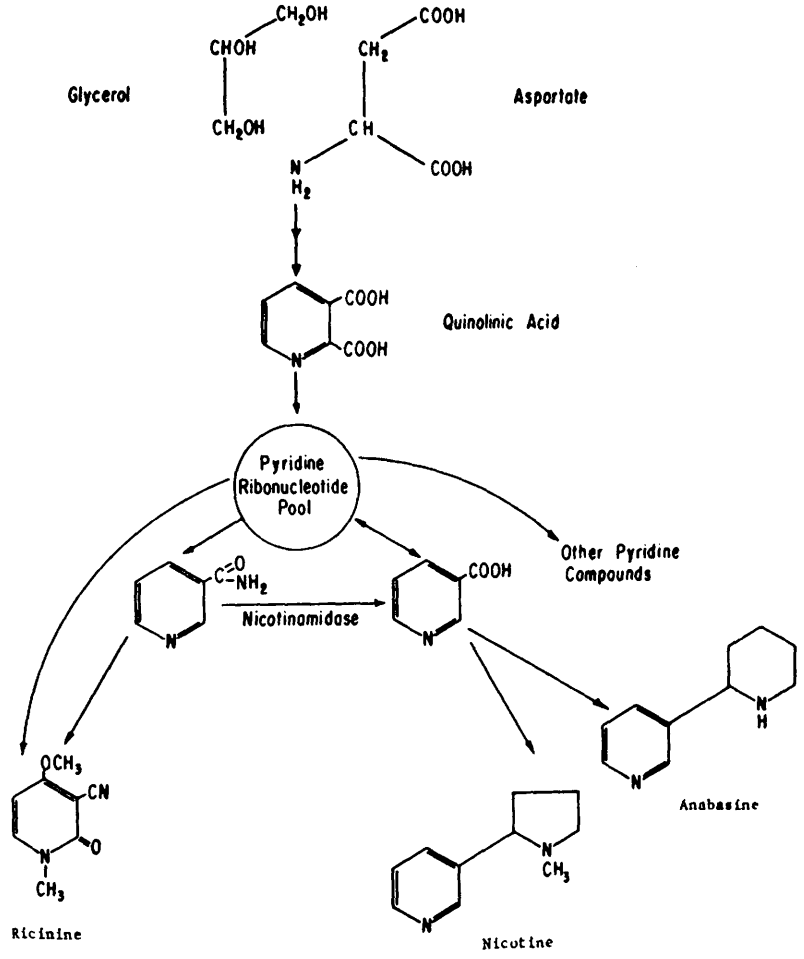

Fig. 1. Pyridine Ribonucleotide Biosynthesis.

Results and Discussion

The specific activities and activities relative to 12 day old seedlings of the various plant tissues studied in the course of this work are presented in Table 1. The results show that 12 day old seedlings had the highest nicotinamidase activity, but that all fractions were active. Flowers and immature seeds were the least active. The R_f of nicotinamide in the solvent system used throughout the course of this work was 0.71. The R_f of nicotinic acid was 0.27. At least four more unidentified products having R_f's of 0.15, 0.20, 0.08 and 0.34 were noted on the chromatograms. These unknown compounds arose from homogenates of female flowers, seeds and flowers and buds. It is possible that these compounds are pyridine nucleotide pool products since it has been shown that nicotinic acid may be converted to NMN and NAD in the castor plant (Young, 1963. Hadwiger *et al.*, 1963; Hadwiger and Waller, 1964).

Table 2 indicates that the nicotinamidase from the castor plant is stimulated by ATP and Mg^{++}.

It is interesting to note that the nicotinamidase from liver (Petrack *et al.*, 1963a, b) does not require a metal cofactor, whereas the enzyme from *Aspergillus niger* does. Since the nicotinamidase from the castor plant is stimulated somewhat by Mg^{++}, it may be similar to the enzyme present in *Aspergillus niger*.

The action of nucleotidase on NAD^+ and $NADP^+$ produces free nicotinamide in the cell. One important biological function of nicotinamidase is to catalyze the formation of nicotinic acid, thus permitting this product to be utilized in the synthesis of pyridine ribonucleotides. Existing evidence (animals, yeast) indicates that nicotinamide cannot be used in the synthesis of the pyridine ribonucleotides. Fig. 1 shows this reaction in relation to the over-all biosynhetic pathway for the pyridine ribonucleotides and ricinine (Yang and Waller, 1964).

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LITERATURE CITED

Curti, B., and G. Porcellati. 1963. Properties of nicotinamide deamidase of pigeon liver extracts. Giorn. Biochim. 12:13.

Hadwiger, L. A., S. E. Badiei, G. R. Waller, and R. K. Gholson. 1963. Quinolinic acid as a precursor of nicotinic acid and its derivatives in plants. Biochem. Biophys. Res. Commun. 13:466.

Hadwiger, L. A., and G. R. Waller. 1964. Unpublished results.

Hughes, D. E., and D. H. Williamson. 1952. The synthesis of cozymase from nicotinic acid and its derivatives by *Lactobacillus arabinosus*. Biochem. J. 51:330.

Joshi, J. G., and P. Handler. 1962. Purification and properties of nicotinamidase from *Torula cremoris*. J. Biol. Chem. 237:929.

Lowry, O., N. Rosebrough, A. Farr, and R. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

Petrack, B., P. Greengard, A. Cranston, and H. Kalinsky. 1963a. Nicotinamide deamidase in rat liver and the biosynthesis of NAD. Biochem. Biophys. Res. Commun. 13:472.

Petrack, B., P. Greengard, A. Cranston, and H. Kalinsky. 1963b. Release of inhibition of rat liver nicotinamide deamidase by hypophysectomy. Biochem. Biophys. Res. Commun. 13:478.

Pichappa, C., and E. Shanmugasundaram. 1962. Studies on nicotinamide deamidase of *Saccharomyces cerevisiae*. Proc. Natl. Inst. Sci. India. B28:432.

Sarma, D., S. Rajalakshmi, and P. Sarma. 1964. Studies on the enzymes involved in nicotinamide adenine dinucleotide metabolism in *Aspergillus niger*. Biochem. Biophys. Acta. 81:311.

Waller, G. R. 1961. Biosynthesis of the alkaloid ricinine in the castor plant *Ricinus communis* L. Ph. D. Thesis. Oklahoma State University.

Waller, G. R., and L. M. Henderson. 1961. Biosynthesis of the pyridine ring of ricinine. J. Biol. Chem. 236:1186.

Yang, K. S. 1963. Biosynthesis of ricinine by *Ricinus communis* L. M.S. Thesis. Oklahoma State University.

Yang, K. S., and G. R. Waller. 1964. J. Biol. Chem. *In Press.*