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THE ROLE OF THE PYRIDINE NUCLEOTIDE CYCLE IN THE BIOSYNTHESIS OF RICININE

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Abstract-Competitive feeding experiments were performed using castor bean seedlings to determine the effect of NAD on the incorporation of radioactive quinolinic acid into the alkaloid ricinine. The exogenous NAD did not cause a decrease in the total radioactivity incorporated into the ricinine as expected, but instead caused an increase. Data previously presented by other investigators was re-examined in light of this new finding. It was concluded that the existing evidence supports the hypothesis that NAD and ricinine are made from quinolinic acid by separate pathways.

INTRODUCTION

NUMEROUS studies have shown that quinolinic acid (2,3 pyridinedicarboxylic acid) is a common precursor to many of the pyridine ring compounds in plants,[1] animals[2] and microorganisms.[3] Among these pyridine compounds are NAD and the other pyridine nucleotide cycle intermediates[4] and the alkaloids nicotine[5] and ricinine.[6]

Leete and Leitz[7] first suggested that intermediates of what is now called the pyridine nucleotide cycle might be involved in the biosynthesis of ricinine in the castor bean plant. Three studies[6,8,9] have been reported which were interpreted as being in support of the hypothesis that this cycle was a necessary intermediate in the biosynthetic pathway between quinolinic acid and ricinine in the castor bean plant, as well as between quinolinic acid and nicotine in the tobacco plant. This conclusion was based on the relative amounts of radioactivity incorporated into ricinine or into nicotine after feeding labeled quinolinic acid, nicotinic acid mononucleotide and NAD to the plants.

The purpose of the present investigation was to test the hypothesis that exogenous NAD is an intermediate in the biosynthesis of ricinine from quinolinic acid. This was accomplished by studying the effect of NAD on the extent of incorporation of quinolinic acid-6-^{14}C into ricinine in ***Ricinus communis*** var. Cimarron.

RESULTS AND DISCUSSION

The effects of the presence of NAD on the incorporation of quinolinic acid-^{14}C into ricinine are shown in Table 1. As can be seen the presence of NAD at a concentration level

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ten times greater than that of quinolinic acid caused no decrease in the incorporation of the quinolinic acid into ricinine but instead, caused an increase. Several hypotheses concerning the role of the pyridine nucleotide pathway and NAD in the synthesis of pyridine ring alkaloids should be discussed in view of the data presented in this and in earlier papers by other investigators.

TABLE 1. THE EFFECT OF EXOGENOUS NAD ON THE INCORPORATION OF RADIOACTIVE QUINOLINIC ACID INTO RICININE

Feeding group	Feeding solution: quinolinic acid-6-^{14}C alone* A	Feeding solution: quinolinic acid-6-^{14}C + NAD† B	B/A	Average B/A
	Total cpm per 100 μl of ricinine solution‡			
I	325	1621	4.9	
II	442	685	1·5	2.9
III	497	1231	2·5	

* 0·17 μ moles in 10 μl solution containing 5 μc per plant.
† 0·17 μ moles of quinolinic acid-6-^{14}C (5 μc) + 1·4 μ moles NAD in 10 μl solution per plant.
‡ After purification and dilution to standard volume (see text).

If one assumes that NAD is an obligatory intermediate in the biosynthesis of ricinine from quinolinic acid and that exogenous NAD can cross the cellular membrane barriers intact, then a ten-fold molar dilution of the radioactive quinolinic acid by NAD should have caused a decrease in the radioactivity incorporated into ricinine (Fig. 1, Pathway II). The data in Table 1 show this is not the case.

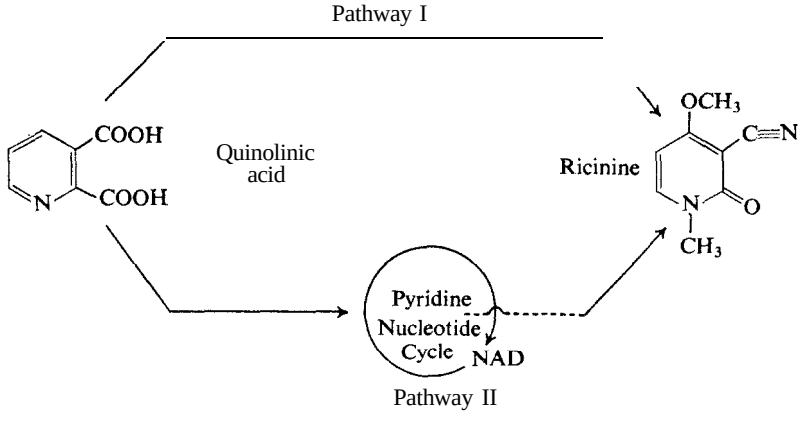

FIG. 1. THE TWO POSSIBLE PATHWAYS FOR THE BIOSYNTHESIS OF RICININE FROM QUINOLINIC ACID.

The pyridine nucleotide cycle operates in much the same manner in a great variety of organisms.[10] Castor beans do not seem to be an exception.[1] Gholson *et al.* found that in

[10] R. K. GHOLSON, Nature 212,933 (1966).

crude extracts NAD acted as an inhibitor of the first reaction in the pyridine nucleotide cycle (the formation of nicotinic acid mononucleotide from phosphoribosylpyrophosphate and quinolinic acid).[2] If NAD or some product of its metabolism crosses into the cells and acts as such an inhibitor in *vivo*, then the large excess (endogenous levels have been reported to be from 10^{-8} to 10^{-9} moles/g of tissue)[11–13] of NAD ($\sim 2 \times 10^{-6}$) given to the experimental plants should have decreased the movement of radioactive quinolinic acid through the pyridine nucleotide cycle. If ricinine and NAD are made by separate pathways (Fig. 1, Pathway I), an inhibition of the pyridine nucleotide cycle could increase the incorporation of ^{14}C from quinolinic acid into ricinine. The data in Table 1 are not inconsistent with this latter hypothesis.

It is true that the stimulation by NAD of radioactive incorporation into ricinine might have been due to some other effect(s) not directly connected with the biosynthesis of the alkaloid or the operation of the pyridine nucleotide cycle. The evaluation, however, of such possible contributing factors as enhancement of transport of quinolinic acid into the cells or general increases in metabolic rates are difficult to evaluate at this time.

Waller *et al.* has shown that exogenous NAD can serve as a precursor of ricinine.[8] Numerous citations show that NAD is hydrolyzed before it can cross into a cell.[14,15] NAD can also act as a source of nicotinamide and nicotinic acid, both of which can be incorporated into ricinine. When equal molar quantities of radioactive quinolinic acid, nicotinic acid, nicotinamide or NAD were fed in separate experiments to castor beans, 10, 5, 3 and 3 per cent respectively of the label was incorporated into the ricinine.[8] Such results suggest that quinolinic acid is a much better precursor for the biosynthesis of ricinine than either of the other three compounds. It seems likely that NAD was incorporated into ricinine as a result of having been first hydrolyzed to nicotinamide or nicotinic acid.

The data of Waller and Henderson[16] concerning the metabolism of nicotinamide in castor beans strongly supports the hypothesis that the pathways of ricinine and NAD biosynthesis from quinolinic acid are separate. They found that the amide nitrogen of exogenous nicotinamide was incorporated intact into the nitrile nitrogen of ricinine. On the other hand, all investigations into the incorporation of nicotinamide into NAD agree that nicotinamide is first metabolized to some form of nicotinic acid beforebeing incorporated into NAD.[2,12,15,17–20] Furthermore, castor beans clearly have an active compliment of enzyme to accomplish the deamidation of nicotinamide.[21] It would seem then from these observations the nicotinamide goes into ricinine and NAD by separate pathways.

The true role of the pyridine nucleotide cycle, if any, in the biosynthesis of ricinine will not be clear until the enzymes involved are completely elucidated. At present, however, the data concerning the percent incorporation of various precursors of ricinine, the amide nitrogen incorporation from nicotinamide and the lack of inhibition by NAD on the incorporation of quinolinic acid into ricinine strongly suggest that NAD and ricinine are made from

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quinolinic acid by separate and more or less independent pathways. Because of the similarities between the biosynthesis of nicotine and ricinine, it would be surprising to find an involvement of the pyridine nucleotide cycle in the biosynthesis of nicotine.[5, 6, 22–25]

EXPERIMENTAL

Materials

Ricinus communis var. Cimarron seeds were a generous gift of Dr. R. D. Brigham, USDA, Lubbock, Texas. Quinolinic acid-6-^{14}C (29.3 μc/μM) was purchased from Nuclear-Chicago.

Procedure

Thirty to forty castor beans were washed with a 1·3 per cent solution of NaOCl and germinated under semisterile conditions at 28" in a media of wet vermiculite contained in covered enamel pots. After 6 days, three plants were selected in the stage of development for maximum ricinine biosynthesis as shown by Robinson.26 Each group of plants (Table 1) consisted of a set of three control plants fed with (quinolinic acid-6-^{14}C alone) and a set of three experimental plants (fed with quinolinic acid-6-^{14}C plus NAD). The eighteen plants which were fed had an average aerial height of 4·8 cm (range 4·0–5·7 cm) and an average aerial weight per group of three plants of 4·5 g (range 4·0–5·0 g). The stem injection procedure of Waller *et al.*[6] was used with the modification that the plants were kept in the dark except during the times of injection of the samples. Each plant received a total of 5 μc of quinolinic acid-6-^{14}C ($1·7 \times 10^{-7}$ moles) in 10 μl of solution (pH 6.8). At zero time each plant was given 7 μl of the solution, 40 min later 2 μl and 20 min later 1 μl. 10 min after the final injection the plants were harvested. The solution fed to half of the plants contained $1·4 \times 10^{-6}$ moles of NAD in addition to the quinolinic acid. At the end of the 70 min feeding period the three plants were removed from the vermiculite and the stems cut at the roots. This aerial portion was quickly weighed and then ground in a Waring Blendor along with approximately 40 g of cold plants to serve as carrier material. The plants were blended twice with 200 ml of hot water and filtered through a course sintered-glass funnel into a suction flask cooled in a water-ice bath. The filtrate was evaporated under reduced pressure to a volume of 100 ml and extracted three times with 100 ml Et_2O. The aqueous phase was filtered through a medium and then fine sintered-glass funnel and the volume reduced to 10 ml. This clear fluid was placed on a Sephadex G-10 column (4 cm by 110 cm), eluted with water at the rate of 2·4 ml/cm^2/hr and collected in 3-ml fractions. The fractions were assayed for u.v. absorption at 260 μm and for radioactivity by liquid scintillation counting. The fractions containing the ricinine were approximately in tubes 475-525. Ricinine was identified by paper chromatography and TLC, using a commercial ricinine standard for comparison, and by u.v. and i.r. absorption spectra. The fractions containing ricinine were pooled, evaporated almost to dryness, transferred to a volumetric flask and diluted to 25 ml. There were sometimes impurities in this ricinine in the form of colored pigments and nicotinamide. To insure that these impurities did not interfere with the determination of the radioactivity in the ricinine, two 100-μl samples were removed and streaked on two 10-cm by 20-cm Silica F_{254} TLC plates. The plates were developed with a solvent system of $CHCl_3$/MeOH/NH_4OH (60: 10: 1) for 1 hr. The ricinine (R_f 0·50) was completely separated from the pigment and the nicotinamide (R_f 0·38). The areas corresponding to ricinine were scraped from the plates, ground to a fine powder in an agate mortar and suspended in 15 ml of Cab-0-Sil/toluene liquid scintillation counting fluid.27 Each sample was counted for six 10 min periods on a Packard Model 3310 Tri-carb Liquid Scintillation Spectrometer.* The total cpm per 100 μl of ricinine solution was taken as a measure of the amount of ricinine made by the plants from quinolinic acid.

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* There was no quenching of the counting efficiency by ricinine up to 1 mg in 5 ml of Bray solution or by up to 200 mg Silica F_{254} in 15 ml of Cab-0-Sil/toluene solution. Both the amounts of ricinine and Silica F_{254} used in each sample were below these values.

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