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## Nicotinic Acid-Ricinine Relationship in Sterile Cultures of *Ricinus communis* L.<sup>1, 2, 3</sup>

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The role of nicotinic acid and nicotinamide as precursors for ricinine, the alkaloid produced by the castor plant, *Ricinus communis* L., is well established (6, 9, 10, 11). Present evidence indicates that the sequence nicotinic acid  $\rightarrow$  nicotinamide  $\rightarrow\rightarrow\rightarrow$  ricinine occurs in the castor plant. The results indicated that the pyridine ring and the amide group are incorporated as a unit into ricinine with the amide undergoing an intramolecular dehydration to give the nitrile. In the course of the present study, it was observed that the ricinine content of the germinating castor seed increased markedly after 48 to 72 hours incubation at 30°. It would appear that a good way to study ricinine biosynthesis would be to add a precursor to the seed or seedling or some of its parts just before this rapid ricinine synthesis occurred. This paper reports the results obtained from studying the incorporation of nicotinic acid-7- $C^{14}$  into ricinine using sterile excised castor embryos, excised cotyledons, and leaf discs. These experiments subsequently led us into a study of ricinine degradation by these tissues. Evidence that ricinine could be metabolized by the castor plant was obtained in 1933 by Weevers (12) when he showed that ricinine disappeared with increasing age of castor plants grown on nitrogen depleted soil.

The experiments reported in this paper establish clearly that a nicotinic acid-ricinine relationship occurs in the castor plant and that the metabolism of ricinine can be spared by the presence of higher concentrations of nicotinic acid in tissue than normally is found.

### Experimental<sup>5</sup>

*Sterile Culture Techniques.* Seeds of *Ricinus communis* L., variety Cimmaron were sterilized with 0.1%  $HgCl_2$ , rinsed with sterile water, and allowed to germinate for 2 days in petri dishes in the dark at 30°. Embryos were excised from the 2-day old seedlings and planted in sterile solid agar medium. They were incubated for 2, 4, 6, and 8 days in the dark at 30°. A typical experiment used 20 embryos in 100 ml of media.

Cotyledons were removed from 7 to 8-day old seedlings grown in the same fashion as described above for the 2-day old seedlings. Twenty to thirty cotyledons were cultured in 25 ml of liquid media for 48 hours at 30°.

Unless specifically mentioned, all cultures were carried out in the dark.

*Culture Medium.* A modified White's (7, 13)

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<sup>5</sup> Some data are reported as ricinine content per embryo, nicotinic acid content per cotyledon, etc. These results are from typical experiments using the number of embryos or cotyledons described. An analysis of the total amount of a component in all of the tissues used in a single experiment was made, and the results are reported on a per unit basis for clarity.

solution was used. Iron sodium ethanol-ethylene-diaminetriacetate at a concentration of 12.5 mg/liter was used as the source of iron. Thiamine (5  $\mu\text{g}/\text{ml}$ ) and pyridoxamine (3  $\mu\text{g}/\text{ml}$ ) were added. For the cotyledon cultures 0.05  $\mu\text{g}/\text{ml}$  of kinetin was added. Sucrose was omitted from the embryo culture media. Nicotinic acid-7- $\text{C}^{14}$  with a specific activity of 9.1 mc/mm was obtained from New England Nuclear Corporation.

**Ricinine Determination.** The castor plant tissue was washed free of culture media with distilled water. The alkaloid was isolated by the procedure similar to that used previously (9). The macerated tissue was extracted three times with chloroform, with a weight ratio of chloroform to fresh plant of 3:1. The chloroform solution was extracted with an equal volume of 7 N  $\text{NH}_4\text{OH}$  and then evaporated to dryness on a steam hot plate. Lipids and pigments were removed from the residue by extraction with ethyl ether. The residue remaining after ether extraction was dissolved in a known volume of distilled water. The ricinine concentration was determined spectrophotometrically by measuring its absorption at 307  $\text{m}\mu$ . In the isolation of radiochemically pure ricinine 10 mg of unlabeled ricinine was added to this aqueous solution. After evaporation to dryness, the ricinine in the residue was then purified to a constant specific activity by sublimation. The melting point was 200.5 to 201.5° C (corrected).

**Nicotinic Acid Determination.** The tissue samples were dried to a constant weight at 80° and pulverized in a mortar and pestle prior to analysis. This material was hydrolyzed with 1 N HCl and autoclaved for 1 hour at 120°. The solution was filtered, diluted, and neutralized with sodium hydroxide. A microbiological assay was done for niacin using *Lactobacillus arabinosus* (4). Growth was measured by titrating the acid produced after a 72 hour incubation period at 37°.

**Isotope Analyses.** The methods described previously were used (9).

## Results & Discussion

The ricinine content of young castor seedlings grown in sand at 30° using tap water was observed to increase 30 to 50-fold in the 2 to 5-day period following planting of the seed. This is the period in the plants' life when the rate of ricinine synthesis is the highest. After the sixth day, the ricinine content was approximately 1.5 mg per seedling. Ricinine synthesis continued as the plants increased in size, but its rate of formation was much slower and it appeared to be dependent on age and physiological development. This period when the most rapid synthesis of ricinine occurred corresponded with the emergence and splitting of the endosperm. Embryos were excised from sterile seedlings grown in agar after 2 days of age which corresponds to the point just prior to rapid alkaloid synthesis. These data confirm and extend the findings of Bottcher (3),

who found that during germination in the dark that the ricinine nitrogen in 100 seeds increased in 3 weeks from 4 to 72 mg and Bogdashevskaya (1), who found that the absolute amount of ricinine increases with the development of the plant.

Figure 1 shows data on the growth of sterile excised embryos which occurred during the 8-day incubation period. Addition of nicotinic acid to the medium had no effect on their growth. When thiamine and pyridoxine were removed, growth was reduced significantly. Experiments were performed to determine if the tissue of the embryos grown in the presence of nicotinic acid contained higher

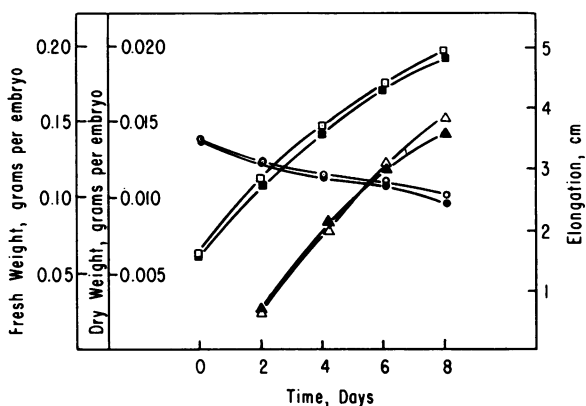


FIG. 1. Growth of sterile excised castor embryos. ○ ●, dry weight; △ ▲, elongation of hypocotyl; □ ■, fresh weight; solid symbols are data obtained when 10  $\mu\text{g}/\text{ml}$  of nicotinic acid was added to the media; open symbols are when no nicotinic acid was added.

quantities of this vitamin than those grown in the medium lacking nicotinic acid. These embryos were analyzed for their total nicotinic acid content. The results in figure 2 clearly show that the tissue concentrations of nicotinic acid were higher when the vitamin was present in the medium. They remained higher than the controls for the entire duration of these experiments.

The rate of disappearance of nicotinic acid from the medium is shown in figure 3. The most rapid uptake was during the first 2 days. After that time, a leveling off period occurred. The slowing down of nicotinic acid uptake did not appear to be due to tissue deterioration. This corresponded to the time when the ricinine content of the embryos was the highest. In the experiments reported in this paper a nicotinic acid concentration of 5 mg/100 ml was used with the result that 60% of the vitamin was taken up by the embryos during the 8-day incubation period.

Figure 2 shows the ricinine content of sterile excised castor embryos. In the experiments where the nicotinic acid was added, a higher ricinine content of the embryos was observed in the 2, 4, and 6 day cultures. After 8 days, no stimulation was ob-

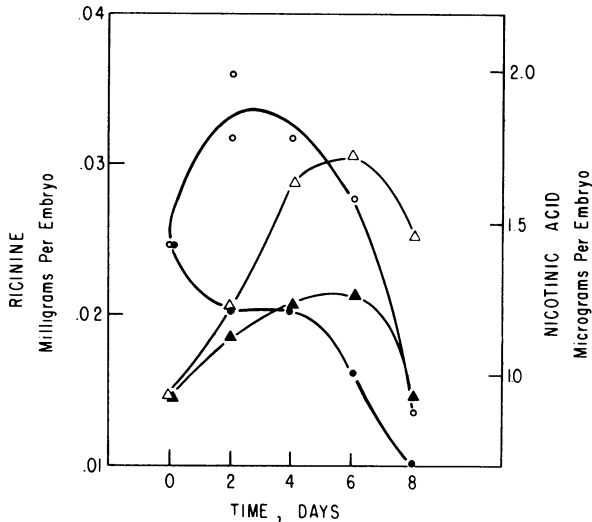


FIG. 2. Ricinine and nicotinic acid content of sterile excised castor embryos. Ricinine: ●, control, ○, nicotinic acid added (5 mg per 100 ml of medium). Nicotinic Acid: ▲, control; △, nicotinic acid added (5 mg per 100 ml of medium).

served. The significance of this inability of the embryos to maintain their high ricinine content when the tissue concentrations of nicotinic acid were significantly higher than normal is not clear. It appears that the utilization of ricinine occurs much faster than does nicotinic acid. This might indicate that ricinine is being metabolized to compounds other than nicotinic acid at a substantial rate. In the absence of nicotinic acid, the ricinine content decreased slowly during the first 4 days and very rapidly during the last 4 day period. The stimulatory effect of nicotinic acid on ricinine formation in castor embryo cultures has also been found in cotyledon cultures, but it has not been observed with whole plants. In whole plants, ricinine formation is constant over a 400-fold range in concentration of

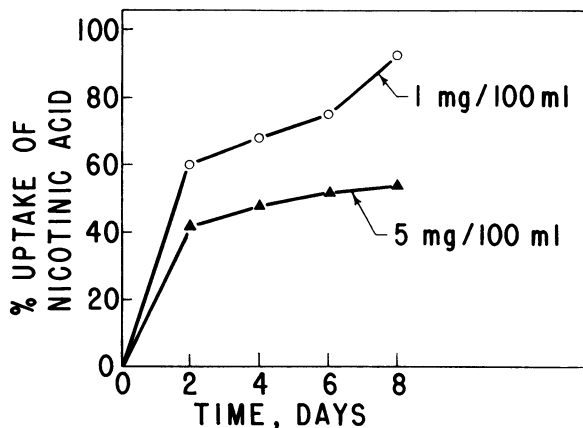


FIG. 3. Uptake of nicotinic acid from the medium by sterile excised castor embryos.

nicotinic acid (9, 10, 11). The dry weight of the embryos decreases linearly over the 8-day culture period; however, the rate at which ricinine disappeared increased rapidly after the first 4 days. In other experiments where sterile embryos were grown for a month, only a trace of ricinine was detected.

The 7 to 8-day old sterile seedlings used for the cotyledon cultures varied from 1.5 to 24 centimeters in length and twofold with respect to fresh and dry weight of their cotyledons. The endosperm of those plants decreased in weight up to tenfold with the increase in seedling size. These results agree with earlier reports (8, 12).

Figure 4 shows the ricinine and nicotinic acid content in the cotyledons of 7 to 8-day old seedlings of different size. The same figure shows the nicotinic acid content of the endosperm of the 7 to 8-day old seedlings. The nicotinic acid of the endosperm decreased as the seedling increased in size, conversely the content in the cotyledon increased. This

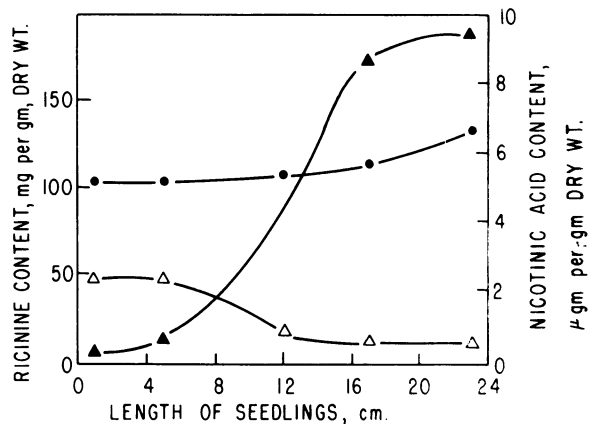


FIG. 4. Ricinine and nicotinic acid of the cotyledons and endosperm of sterile 7 to 8 day old castor seedlings. ▲, ricinine content of cotyledons; ●, nicotinic acid content of cotyledons; △, nicotinic acid content of endosperms.

might be interpreted as supporting James's (5) theory that precursors of ricinine are present in the endosperm, and that ricinine is formed as a result of the translocation of these precursors from the endosperm to the cotyledon. It is not clear if an increase in nicotinic acid in the cotyledons represents de novo synthesis in the cotyledons or if it represents a translocation from the endosperm to the cotyledon.

When nicotinic acid was added to the medium, there was no increase in the nicotinic acid content of either the cotyledon or the hypocotyl. These results shown in table I indicate that the normally developing plant does not require an external source of nicotinic acid.

The ricinine content of the cotyledon, hypocotyl, root, and endosperm was determined on seedlings which were grown in the presence of nicotinic acid and in the absence of nicotinic acid. The results are

Table I

*Distribution of Ricinine & Nicotinic Acid in 7 to 8 Day Old Sterile Castor Seedlings*

	Ricinine		Nicotinic acid	
	Control	Nicotinic acid added*	Control	Nicotinic acid added*
	$\mu\text{M/g fr wt}$		$\mu\text{M/g dry wt}$	
Cotyledon	29.3	30.5	0.92	0.90
Hypocotyl	3.7	3.9	0.86	0.95
Root	1.3	1.3	...	...
Endosperm	Trace	Trace	...	...

\* Fifty  $\mu\text{g/ml}$  of nicotinic acid was added to the medium.

shown in table I. The ricinine content was highest in the cotyledons which agrees with the report by Robinson and Fowell (8). There was no stimulation of ricinine formation when nicotinic acid was added to the medium. This confirms the rate constancy of ricinine synthesis when nicotinic acid is added to whole castor plants (9). Only a trace of ricinine was detected in the endosperm of the seedlings at their different stages of development which agrees with the previous reports (3, 8, 9).

Cotyledons from sterile plants over 8 cm in length were removed for separate experiments to determine their nicotinic acid-ricinine relationship. Cotyledons of uniform size selected from the same size plants were used for a given experiment. After 48 hours incubation, a twofold increase in dry weight and a threefold increase in fresh weight was observed. The dry weight increased about 10% more when the cotyledons were grown in the light than when they were grown in the dark. An approximate three to fourfold increase in the surface area of the cotyledons was observed.

Table II

*Ricinine & Nicotinic Acid Content of Sterile Excised Cotyledons*

Time, hours	Ricinine		Nicotinic acid	
	Control	Nicotinic acid added*	Control	Nicotinic acid added*
	$\mu\text{moles/cotyledon}$			
0	0.67	0.67	0.020	0.020
48, dark	0.07	0.79	0.025	0.12
48, light	0.70	0.87	0.021	0.13

\* Fifty  $\mu\text{g}$  per ml of nicotinic acid was added to the medium.

It may be seen in table II that no significant net synthesis of either ricinine or nicotinic acid occurred in the cotyledons during the 48 hour incubation period under the conditions used in these experiments. An occasional experiment did result in 25 to 50% net synthesis of ricinine in the light. The cause for this variation is not known; however, it is thought to be due in part to the variation in the castor seeds used in this study. The constancy of nicotinic acid content of whole castor plants grown in media with added nicotinic acid is in sharp contrast to the higher nicotinic acid content of cotyledons grown in media

containing added nicotinic acid. The tissue concentrations were increased sixfold, and this was not affected by light. By comparison ricinine was rapidly utilized by sterile castor cotyledons in the dark, only 10% of the original amount remaining. After incubation, the media was found to contain approximately the same amount of ricinine as the cotyledons. Considerable variability in the extent of ricinine utilization by the cotyledons was observed among different experiments. The results usually were in the range of 20 to 90% decrease of ricinine in the castor cotyledons. Correcting for the ricinine in the media would give a range of 10 to 45% of the original ricinine which had been utilized by the cotyledons. It has been reported (2) that an 18% reduction in the ricinine content occurs in leaves of plants which were shaded from the light; however, the upper unshaded leaves of such plants produced ricinine at a 14% higher level than normal indicating that no significant change in total alkaloid content occurred. The results previously discussed have also shown conclusively that ricinine is metabolized by the sterile growing excised castor embryo in the dark. When nicotinic acid was added to the medium, this utilization of ricinine in the dark by the cotyledons was not observed. This suggests that nicotinic acid has a sparing action upon ricinine utilization by the cotyledon. It can be seen in table II that the tissue concentrations of nicotinic acid are about five to sixfold higher when the vitamin has been added to the medium; however, the nicotinic acid content in the control cultures is not changed. It may be inferred from these data that in periods of stress ricinine was converted to nicotinic acid or an intermediate compound related to both nicotinic acid and ricinine and that the alkaloid could end up as a part of the pyridine nucleotide fraction of the cotyledons. In vitro studies on ricinine degradation are being conducted which may help in clarifying this nicotinic acid-ricinine relationship.

Evidence (unpublished) has been recently obtained that in the whole plant tritium labeled ricinine is converted to tritium labeled nicotinic acid. These results are under further investigation.

To study the biosynthesis of ricinine by sterile embryos and cotyledons, nicotinic acid-7- $\text{C}^{14}$  was added to the medium at the beginning of the incubation period. The extent of its incorporation into ricinine by these tissues is shown in table 3. The results from incorporation studies using these sterile cultures are compared to similar studies using whole castor plants. It should be noted that the embryo experiments report the incorporation of nicotinic acid-7- $\text{C}^{14}$  after 96 hours which was the period when maximum ricinine synthesis had occurred. These experiments indicate that embryos and cotyledons are not as effective in forming ricinine from nicotinic acid as the whole castor plant. Castor plants which have been injected with nicotinic acid are much more efficient in carrying out ricinine biosynthesis from this precursor than ones grown in liquid cultures containing added nicotinic acid (10). The availability

**Table III**  
*Incorporation of Nicotinic Acid-7-C<sup>14</sup> into Ricinine*

	Duration of experiment	Nicotinic acid-7-C <sup>14</sup> fed				Total amount of radioactivity recovered in ricinine	Incorporation into ricinine*
		Quantity	Uptake	Specific activity	Radioactivity taken up by tissue		
	hr	$\mu$ moles	%	$\mu$ mc	$\mu$ mc	$\mu$ mc	%
Embryo culture**	96	40.7	87.4	109	3,884	2.33	0.06
Cotyledon culture	48	10.2	97.4	75.5	750	1.13	0.15
Whole seedlings*** (Cotyledenous stage; Nutrient solution culture)	72	0.71	65.0	5,880	2,713	75	0.28
Whole plant† (7th nodal stage; Injection technique)	96	0.28	...	5,880	1,667	530	31.8

\* Percentage of incorporation was determined by dividing the total activity of the ricinine isolated by the activity taken up by the plant tissue.

\*\* Grown in the dark. All other experiments were conducted in the sunlight.

\*\*\* Forty seedlings were used in this experiment.

† Single plant experiment.

of nicotinic acid within the cell would appear to be an important factor in ricinine biosynthesis. In these sterile cultures the tissue concentrations of nicotinic acid were higher than average.

Sterile leaf disc cultures were investigated briefly to determine if they would form ricinine from nicotinic acid-7-C<sup>14</sup>. Only a trace of incorporation (less than 0.001%) of radioactivity into ricinine was observed. Young and old castor leaves were used in these experiments and no difference in their ability to grow or to synthesize ricinine was observed.

### Summary

Ricinine biosynthesis and degradation has been studied using sterile embryo and cotyledon cultures from the castor plant. The incorporation of radioactivity from nicotinic acid-7-C<sup>14</sup> into ricinine was low when these parts of the plant were used; however, it was of the same order of magnitude as was obtained from feeding the precursor to whole castor plants in nutrient solution.

An increase in the ricinine content of sterile cultures of embryos and cotyledons was observed when nicotinic acid was added to the medium; however, whole plants grown in sterile media were not affected by added nicotinic acid.

The utilization of ricinine in sterile excised cotyledons was found to occur more rapidly in the dark than in the light. When nicotinic acid was present in the medium, no decrease in ricinine content occurred. This sparing action of nicotinic acid on ricinine utilization suggests a vitamin-alkaloid metabolic relationship not previously found in a plant system.

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