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ENZYMES OF ALKALOID BIOSYNTHESIS—I.
ENZYMATIC OXIDATION OF 1-METHYLNICOTINONITRILE

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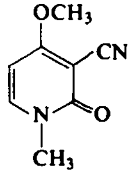
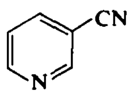
Abstract—A crude enzyme system prepared from young castorbean seedlings catalyzes the oxidation of 1-methylnicotinonitrile to the corresponding 4- and 6-pyridones. The implications of this reaction for the biosynthesis of the alkaloid ricinine are considered. Other pyridone alkaloids might be formed by similar reactions.

INTRODUCTION

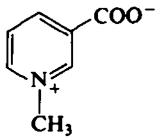
IT HAS been well demonstrated by isotopic tracer experiments that the alkaloid ricinine of the castorbean plant (*Ricinus communis* L.) is biosynthetically derived from nicotinamide.[1] However, the enzymatic details of this conversion remain obscure. Possible intermediates might be nucleotide or 1-methyl derivatives of nicotinamide or nicotinonitrile. By testing a crude enzyme preparation from the castorbean plant for catalytic activity against several of these suggested precursors, it was hoped to find some support for their involvement.



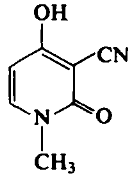
Ricinine



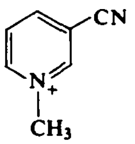
Nicotinonitrile



Trigonelline



Ricininic acid



1-Methylnicotinonitrile

RESULTS

Since the enzymes required for ricinine synthesis are presumably most active at the time and in the part of the plant where the ricinine concentration is increasing most rapidly, analysis of plant parts for ricinine content was carried out by spectrophotometric assay, and results are summarized in Fig. 1. At the time of most rapid growth, ricinine content was increasing at the greatest rate. Dissection of the plant and analysis of separate organs revealed that little or no alkaloid was present in the root. The highest concentration was observed in endosperm, cotyledons and upper hypocotyl with intermediate amounts in the lower hypocotyl. These analytical results agree with findings of several other workers.[2,3]

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[1] G. R. WALLER and L. M. HENDERSON, *J. Biol. Chem.* 236, 1186 (1961).

[2] G. R. WALLER and K. NAKAZAWA, *Plant Physiol.* 38, 318 (1963).

[3] U. SCHIEDT, G. BOECKH-BEHRENS and A. M. DELLUVA, *Z. physiol. Chem.* 330, 46 (1962).

A crude, dialyzed enzyme preparation obtained from the aerial portions (hypocotyl, cotyledons, endosperm) of 6–10-day-old castorbean plants was tested for dehydrogenase activity against several possible intermediates using methylene blue or triphenyltetrazolium chloride as hydrogen acceptors. The results are summarized in Table 1. The only one of these compounds which served as a substrate at an appreciable rate was 1-methylnicotinonitrile (as iodide or perchlorate salt). By a spectrophotometric assay procedure with this substrate the appearance of new absorption peaks at 255 mμ and 285 mμ was observed during the course of enzymatic reaction. Using either the quantitative tetrazolium procedure or the spectrophotometric assay the relation of activity to pH was determined as shown in Fig. 2. The drop in activity at high pH values resulted from irreversible inactivation of the enzyme so that the peak at 9·5 does not represent a pH optimum in the usual sense. The enzyme was also irreversibly inactivated below pH 5·0.

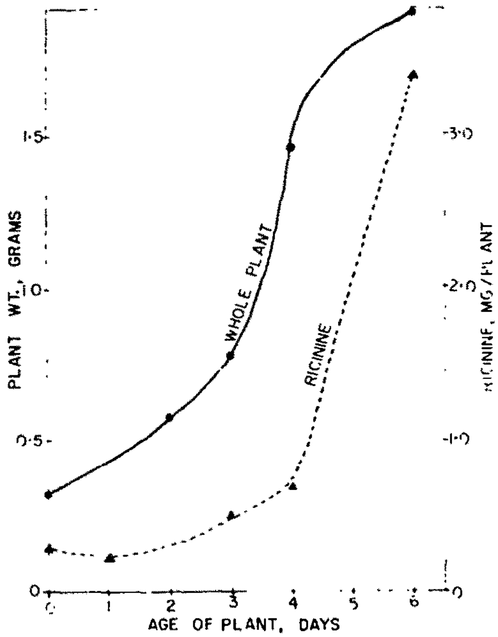

FIG. 1. VARIATION IN FRESH WEIGHT AND RICININE CONTENT OF CASTORBEAN SEEDLINGS WITH AGE OF PLANT

TABLE 1. POSSIBLE RICININE INTERMEDIATES TESTED BY TETRAZOLIUM ASSAY PROCEDURE

Substrate	10% Dye reduction time
1-Methylnicotinonitrile	Within 30 min
1-Methylnicotinamide	Within 4 hr
NAD	Within 4 hr
Nicotinamide	Not within 24 hr
Nicotinonitrile	Not within 24 hr
Trigonelline	Not within 24 hr

Enzyme preparations made at different stages of plant growth were tested for activity by the tetrazolium procedure. No activity was apparent up to 4 days after planting. Slight activity appeared on the fifth day, increased greatly on the sixth day, and reached a plateau on the seventh day. The activity then continued at about the same level up to 2 weeks. Preparations made from leaves of mature castorbean plants grown in the greenhouse showed little or no activity. Root homogenates were very dark colored so that it was not possible to measure enzyme activity in them by our methods. There is no reason to believe that the enzyme is particle bound. Centrifugation at 198,000 *g* for 2 hr left all activity in the supernatant solution. Mitochondria prepared by the procedure of Beevers and Walker[4] showed no activity against 1-methylnicotinonitrile.

Isolation of products from large-scale enzymatic reaction mixtures by chloroform extraction and column chromatography techniques gave two crystalline compounds with some indication of a small amount of a third compound which was more strongly absorbed on the cellulose-powder column. The spectra of the two crystalline compounds were very similar to each other and resembled those of the 4- and 6-pyridones of 1-methylnicotinamide.[5]

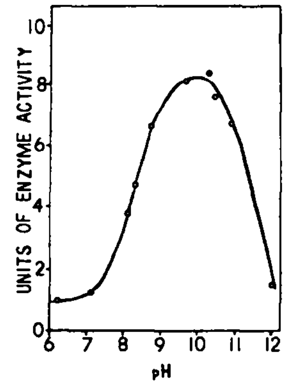

FIG. 2. ENZYME ACTIVITY VS. pH, SPECTROPHOTOMETRIC ASSAY.

For detailed comparisons with the compounds isolated from the enzyme reaction mixture the three different pyridones and ricininic acid were synthesized,[6,7] and the two crystalline products shown to be identical to the 4- and 6-pyridones of 1-methylnicotinonitrile. Not enough of the third compound was available for detailed studies, but its u.v. absorption spectrum was very similar to that of ricininic acid (Fig. 3). Even smaller amounts of still other substances have been indicated by paper chromatography of extracts from the enzymatic reaction mixture, but they have not been studied further.

The specificity of the crude enzyme preparation was investigated using a number of other substrates, testing all of them by the tetrazolium assay and starred ones also by the spectrophotometric assay: 1-methylnicotinamide iodide*, trigonelline, acetaldehyde, hypoxanthine, nicotinamide adenine dinucleotide* (NAD), nicotinamide adenine dinucleotide phosphate, pyridine methiodide, quinoline methodide, iso-quinoline methiodide, acridine methochloride, quinine sulfate, xanthine, berberine sulfate, sanguinarine sulfate*, 1-ethyl-nicotinonitrile bromide*, 1-benzylnicotinonitrile chloride*, 1-methyl-3-cyano-4-pyridone*, 1-methyl-3-cyano-6-pyridone*. Of these compounds the only ones found to react at an appreciable rate were the 1-ethyl and 1-benzyl derivatives of nicotinonitrile. Assuming the molar extinction

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[4] H. BEEVERS and D. A. WALKER, *Biochem. J.* 62, 114 (1956).

[5] M. L. W. CHANG and B. C. JOHNSON, *J. Biol. Chem.* 234, 1817 (1959).

[6] T. ROBINSON and C. CEPURNEEK, *Phytochem.* 4, 83 (1965).

[7] L. MAQUENNE and L. PHILIPPE, *Compt. rend.* 138, 506 (1904).

coefficients for the reaction products to be approximately the same for all substrates of the enzyme, the reaction rates for the 1-ethyl and 1-benzyl compounds were estimated as respectively ½ and ⅔ the rates with 1-methylnicotinonitrile. By the tetrazolium assay 1-methylnicotinamide and NAD showed very slight activity, but no spectral changes were observed when they were used as substrates in the spectrophotometric assay. None of the other compounds tested showed any activity. There was no indication of any cofactor requirements for enzyme activity. Prolonged dialysis (up to 120 hr) did not result in significant loss of activity more than was lost in ordinary storage for this time. However, if the dialysate was concentrated *in vacuo* and added back to enzyme assay mixtures a small stimulation of activity was sometimes noted by both assay procedures. Tetrazolium reduction proceeded more rapidly in evacuated tubes than in air; but if dye acceptor was omitted, the spectral changes noted above occurred only in the presence of air. Addition of $NADH_2$, ascorbic acid, or boiled plant extracts to the tetrazolium assay increased the rate of reduction in air but had no effect under evacuated conditions. These additions are presumed to produce their effect by removing oxygen from the system as a result of their enzymatic or non-enzymatic oxidation.

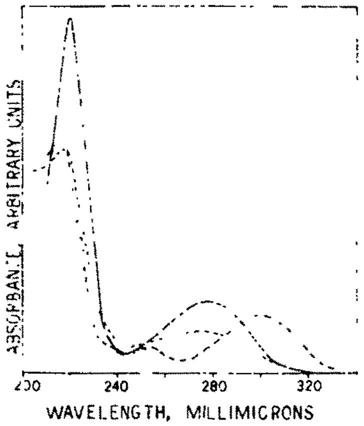

FIG. 3. COMPARISON OF ABSORPTION SPECTRUM OF PEAK C FROM CELLULOSE POWDER COLUMN WITH KNOWN RICININIC ACID.

——— Known ricininic acid in water
- - - - Unknown C in water
. . . . . . Known ricininic acid in N HCl
– · – · – · Unknown C in N HCl

DISCUSSION

The presence of an enzyme in castorbean seedlings which appears to catalyze specifically the oxidation of 1-alkyl-3-cyanopyridinium salts to corresponding 4- and 6-pyridones is of interest because of the possible relationship of this reaction to the biosynthesis of ricinine. Although detailed purification and characterization of the enzyme have not been completed, it is apparently different in activity from enzymes which catalyze similar reactions in other organisms[8-11]. In particular its lack of activity against acetaldehyde or quinine distinguishes it from the aldehyde oxidase or quinine oxidase of liver which appears to be responsible for

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[8] S. CHAYKIN, *Biochim. Biophys. Acta* **82**, 633 (1964).

[9] K. DECKER, F. A. GRIES and M. BRÜHMÜLLER, *Z. physiol. Chem.* **323**, 249 (1961)

[10] G. G. VILLELA, *Enzymologia* **25**, 261 (1963).

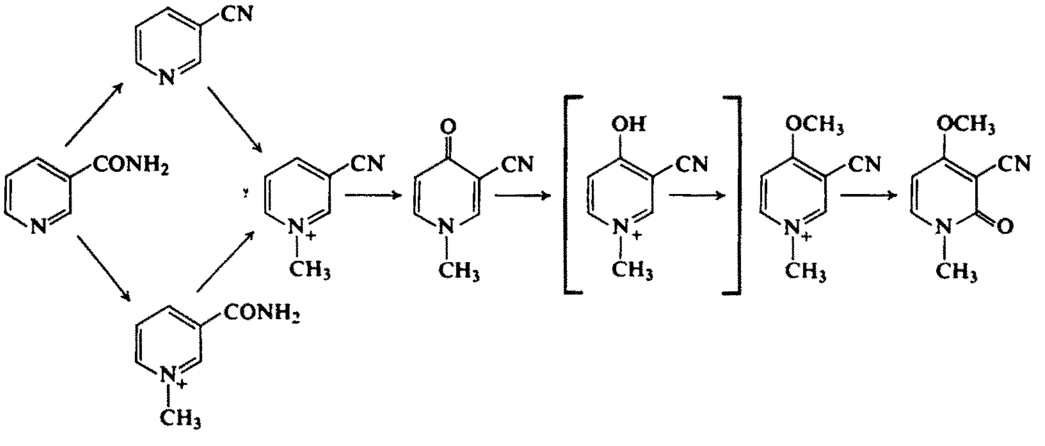
[11] L. GREENLEE and P. HANDLER, *J. Biol. Chem.* **239**, 1090 (1964).

synthesis of the 4- and 6-pyridones of 1-methylnicotinamide.[8] Its association with the parts of the plant which are most actively accumulating ricinine is suggestive of its participation specifically in the ricinine pathway.

If the enzymatic oxidation of 1-methylnicotinonitrile is to be incorporated into the metabolic pathway leading from nicotinamide to ricinine, either nicotinonitrile or 1-methylnicotinamide would appear to be likely intermediates. In some preliminary trials we have had no success in showing either enzymatic methylation of nicotinonitrile or conversion of nicotinamide to nicotinonitrile, so the choice between these two alternatives remains open. The possibility that more complex pyridine derivatives such as the nucleotides may be the real intermediates was first suggested by Leete and Leitz[12] and implied in more recent work by Hadwiger *et al.*[13] It has not been possible to obtain a ribonucleotide of nicotinonitrile such as the 3-cyano analogue of NAD, but since our enzyme preparation does not seem highly specific for the 1-alkyl group, such a nucleotide might well be oxidized and, if so, could be the true intermediate.

The formation of 6-pyridone raises other problems since from its structure it cannot lead to ricinine or to other compounds known to be present in the castorbean plant. If the 6-pyridone is not purely an *in vitro* artifact, it might be completely broken down to simple products in the plant while the 4-pyridone goes on to ricinine.

Since 1-methyl-3-cyano-4-pyridone is stable for many hours in enzyme preparations which actively oxidize 1-methylnicotinonitrile, and since no 2-pyridone has ever been observed as a product of the enzymatic oxidation of 1-methylnicotinonitrile, the introduction of oxygen at the 2-position of ricinine remains a mystery. Methylation of the 4-pyridone at the oxygen to form a 1-methyl-3-cyano-4-methoxypyridinium salt might open the way to oxidation of the ring at the 2-position to form ricinine. This oxidation might even be non-enzymatic since attack at the 2-position seems to be preferred in chemical oxidation.[6] We have attempted to synthesize 1-methyl-3-cyano-4-methoxypyridinium iodide without success. As observed by Wieland *et al.*,[14] this compound readily decomposes to the 4-pyridone. However, it could still be a transient intermediate *in vivo*. Summarizing the above discussion, the following pathway is proposed tentatively for the biosynthesis of ricinine:



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[12] E. Leete and F. H. B. Leitz, *Chem. & Ind. (London)* 1572 (1957).

[13] L. A. Hadwiger, S. E. Badiei, G. R. Waller and R. K. Gholson, *Biochem. Biophys. Res. Comm.* **13**, 466 (1963).

[14] T. Wieland, C. Fest and G. Pfleiderer, *Ann.* **642**, 163 (1961).

There are further implications of the present findings for alkaloid biosynthesis in general. Very few investigations have been made of the enzyme systems specifically involved in formation of alkaloids—probably because it is not obvious how to look for such enzymes. The discovery of an enzyme which forms pyridones from pyridinium salts suggests that other pyridone alkaloids might be produced in the same way. For example, mimosine, lunine, echinopsine, edulein, and most of the acridine alkaloids are 4-pyridones. Flindersine, cytisine, anagyrine and oxysanguinarine are 2-pyridones. The last example is particularly interesting since oxysanguinarine and sanguinarine occur together in *Sanguinaria* spp.; and the latter is pyridinium salt which can be chemically oxidized to the former.[15] Although the castorbean enzyme does not catalyze this oxidation, it would be useful to examine *Sanguinaria* for the presence of an enzyme which does. Oxidation to a 2-pyridone might also be an intermediate step in the conversion of the pyridinium alkaloid berberine to hydrastine.[16] Franck[17] has postulated the participation of quaternary bases as alkaloid precursors, and the present report suggests a specific example of such participation.

EXPERIMENTAL

Plants

Castorbean seeds (*Ricinus communis* var. Cimarron) were germinated in vermiculite kept moist with tap water. The trays in which they were grown were kept covered with aluminum foil but no special precautions were taken to exclude light all the time.

Chemicals

The various N-alkylated compounds used as substrates were synthesized by published methods with slight modifications.[18–20] Preparation of the known pyridones of 1-methyl-nicotinonitrile is described in the following paper.[6] Other compounds were obtained commercially.

Ricinine Analysis

Ricinine was determined spectrophotometrically by extracting plant tissue with chloroform as in the procedure of Waller and Nakazawa[2] except that with the young, etiolated plants there was no interference by other substances, and direct measurement of absorbance at 320 mμ in the chloroform extract was possible. However, it appeared that the younger the plant was, the smaller the fraction of total ricinine that was extractable with cold chloroform. Thus, the use of direct chloroform extraction, while indicating the time of greatest increase, exaggerates the percentage of increase. For a more accurate measurement of total ricinine, macerated plant tissue was extracted with boiling water and the ricinine then extracted from the aqueous extract with chloroform. In dormant seeds the boiling-water treatment released 0·28 mg ricinine/plant (Fig. 1) whereas direct chloroform extraction showed only 0·017 mg/plant. By the time the plants were 6 days old, direct chloroform extraction showed 70 per cent as much ricinine as did the boiling-water method. The nature of the "bound ricinine" is completely unknown.

Preparation of Enzyme Solution

Freshly harvested plant material was homogenized in a chilled Waring Blendor with an equal weight of cold 0·1 M K_2HPO_4 for less than 30 sec. The homogenate was strained through

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[15] E. SPATH, F. SCHLEMMER, G. SCHENCK and A. GEMPP, *Ber.* **70**, 1677 (1937).

[16] J. R. GEAR and I. D. SPENSER, *Can. J. Chem.* **41**, 783 (1963).

[17] B. FRANCK, *Angew. Chem.* **74**, 724 (1962).

[18] J. W. HUFF and W. A. PERLZWEIG, *J. Biol. Chem.* **150**, 395 (1943).

[19] P. KARRER and F. J. STARE, *Helv. Chim. Acta* **20**, 418 (1937).

[20] L. BENDA, *Ber.* **45**, 1787 (1912).

two layers of cheesecloth and adjusted to pH 8·5 with NaOH. The homogenate was then centrifuged at 0° for 30 min at 24,000 *g*. The fatty top layer and sediment were removed and the supernatant solution dialyzed in the cold against 0·01 M K_2HPO_4 (usually for 24–36 hr with several changes of medium).

Tetrazolium Assay

Each assay tube contained: 0·8 ml enzyme solution; 0·2 ml sodium bicarbonate buffer 0·5 M, pH 9·5; 0·1 ml triphenyltetrazolium chloride 0·01 M; 1 μmole desired substrate; and water to 1·6 ml.

Blanks omitting enzyme and omitting substrate and standards with graded levels of triphenyltetrazolium chloride reduced with dithionite for visual comparison of color were run simultaneously. All tubes were covered with a layer of mineral oil to exclude air and the times for 10, 20, and 40 per cent reduction of the dye noted. In certain experiments the effect of other additions was noted by replacement of some of the water with the desired solution.

Spectrophotometric Assay

The Beckman DB double-beam spectrophotometer was used. This instrument permits placing two cuvettes each into the reference and sample beams. Additions were made to four 1 cm^2 cuvettes as follows:

	A	B	C	D
		(ml)		
0·5 M HCO_3^- buffer pH 9·5	0·2	0·2	0·2	0·2
5×10^{-4} M substrate	0·3	—	—	0·3
Enzyme solution	—	0·05–0·3	—	0·05–0·3
Water to 3·0 ml				

After starting the reaction by adding enzyme to cuvette D, A and B were put in the reference beam, C and D in the sample beam; and the appearance of product was followed by observing increase in absorbance at 255 mμ for 10-min intervals over a period of 30 min. Alternatively, the complete difference spectra were plotted over the range 240–340 mμ at each time-interval. The amount of enzyme assayed was chosen to give a change in absorbance of less than 0·15 in 10 min. This amount gave a reaction linear with time up to 30 min. The average absorbance change in 10 min × 100 was defined as units of activity. For determination of the variation of activity with pH, the hydrogen ion concentration was adjusted in unbuffered solutions using HCl or NaOH.

Isolation of Products

To 150 ml of enzyme solution was added 50 mg of 1-methylnicotinonitrile iodide dissolved in a little water. The pH of the mixture was adjusted to 9·0 and the solution swirled intermittently for 1 hr. At the end of this time enough anhydrous sodium sulfate was added to give a pasty mass which was extracted by grinding it with three 50 ml portions of chloroform. Chloroform was removed from the extract by distillation from the steam bath and traces of castor oil removed from the residue by extraction with warm cyclohexane. The dry residue was dissolved in 3 ml of 95% ethanol, made into a slurry with cellulose powder (Whatman Standard), and the ethanol allowed to evaporate. A column 20 × 1·2 cm was prepared of cellulose powder slurried with water-saturated iso-amyl alcohol. The cellulose powder containing compounds to be separated was slurried with some of the same solvent and packed on top of the column. The column was eluted with this solvent and 120-drop fractions collected. Measurement of absorbance at 255 mμ showed a concentration of material in fractions 6–8, 9–16 and 24–29. There was some overlapping in fractions 8 and 9 but clean separation was achieved by the repassage of each group of fractions through the column. The first two peaks from the column, designated A and B in order of appearance, were separately evaporated to dryness at 60° in a rotary evaporator and the residues crystallized from hot ethanol. By comparison with melting points, u.v. and i.r. spectra, and R_f values on paper chromatograms of known compounds A was found to be identical with 1-methyl-3-cyano-6-pyridone and B with 1-methyl-3-cyano-4-pyridone (see following paper for details of the constants). The third peak (C) was present in much smaller amount and showed a single absorption peak at 278 mμ in water or basic solution and shifts in acid.

Note added in proof. R. Mukherjee and A. Chatterjee[21] have shown that a new alkaloid found in *Trewia nudiflora* and named nudiflorine is 1-methyl-3-cyano-6-pyridone, one of our enzymatic products. This is especially interesting since *Trewia* and *Ricinus* are both in the Euphorbiaceae.

Acknowledgements—I want to thank the National Science Foundation and the Research Corporation for grants in support of this research and Mr. John E. Bear of the U.S. Department of Agriculture for the castor-bean seeds.

[21] R. MUKHERJEE and A. CHATTERJEE, *Chem. & Ind. (London)* 1524 (1964).