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PYRIDINIUM OXIDASES IN THE FAMILY EUPHORBIACEAE

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Abstract—At least seven species (*Chrozophora plicata*, *Jatropha gossypifolia*, *Ricinus communis*, *Tragia involucrata*, *Trewia nudiflora*, *Acalypha diversifolia*, *Synadenium grantii*) in the family Euphorbiaceae have shown enzymatic oxidizing activity on salts of 1-methyl-nicotinonitrile. 4- and 6-pyridones were shown to be the enzymic products *in vitro* for both *Trewia nudiflora* and *Ricinus communis*.

INTRODUCTION

The biosynthesis of the pyridine alkaloid ricinine in castor bean plants (*Ricinus communis*, Euphorbiaceae) has been studied extensively by isotopic tracer experiments.[1-8] However, practically no information is available on the enzymatic system or on the detailed steps responsible for the conversion of a particular pyridine compound to ricinine. An attempt has been made in this laboratory to investigate the alkaloid formation at an enzymatic level. In a previous paper,[6] Robinson reported the discovery of a crude enzyme preparation which appeared to catalyze the oxidation of salts of 1-alkylnicotinonitrile to the corresponding 4- and 6-pyridones in the castor bean plants. This finding suggested that pyridinium oxidase might be involved in ricinine biosynthesis, and that the 4-pyridone might likely be an immediate precursor of ricinine.[6] Recently both labeled free nicotinonitrile and 1-methyl-nicotinonitrile have been shown not to be incorporated into ricinine.[5] In addition, the finding of low specificity of the pyridinium oxidase for 1-alkyl substituent suggested that the pyridinium oxidase system may act *in vivo* on some 1-substituted derivative of nicotinonitrile such as nucleotide or nucleoside.[6,9] The finding of an alkaloid nudiflorine in *Trewia nudiflora*,[10]

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[8] T. Robinson, *The Biochemistry of Alkaloids*, Springer-Verlag, Berlin (1968).
[9] P. Fu, Ph.D. Dissertation, University of Massachusetts (1969).
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which is identical to 1-methyl-3 cyano-6-pyridone formed in the pyridinium oxidase system of *Ricinus communis*, and the reported presence of ricinine in *Crotontiglium*[11] suggested that a common pyridone forming enzyme might exist among members of the family Euphorbiaceae. A comparative study was thus designed to investigate the specificity of this enzymatic system among plants of the Euphorbiaceae as well as other plants.

RESULTS

A survey of various plants such as beans, barley, tobacco, sunflower, orange, lemon and squash showed no pyridinium oxidizing activity in crude enzyme preparations.[12] The possible occurrence of this enzymatic system among plants in the Euphorbiaceae was then studied. From a group of eleven species of this family, at least seven were shown to have enzymatic

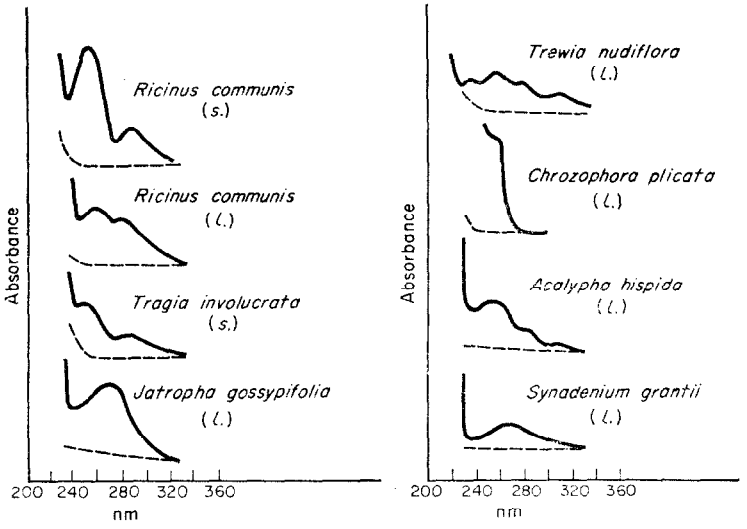

FIG. 1. U.V. SPECTRA OF PYRIDINIUM OXIDIZING ENZYMATIC SYSTEM OF VARIOUS MEMBERS OF THE EUPHORBIACEAE.

s., Seedling (aerial portion); l., leaves.

activity on salts of 1-methylnicotinonitrile. The enzyme assay was done on the extracts of seedlings, sometimes on leaves and on whole plants. There was no attempt to isolate any alkaloids from these plants, since only a very small quantity of material was available. Spectra obtained in the enzymatic reaction from these species are shown in Fig. 1. Strikingly similar spectra between *Ricinus communis* and *Tragia involucrata* indicate the presence of a similar enzymatic system. The differences in spectra among the rest may result from more than one enzymatic system or a sequence of enzymatic reactions being involved in the process of pyridinium salt oxidation. The presence of a pyridone-forming enzymatic system in *Trewia nudiflora* correlates with the occurrence in it of the pyridone alkaloid nudiflorine.[9,10] Since this enzymatic activity was not found in unrelated plants, the present findings suggest the possibility of a family-specific enzyme system.

[11] J. J. WILLAMAN and B. G. SCHUBERT, *Alkaloid Bearing Plants and Their Contained Alkaloids*, p. 84, Technical Bulletin No. 1234, U.S. Department of Agriculture (1961).
[12] P. FU, M.S. Thesis, University of Massachusetts (1966).

Previous studies[6] suggested that castor bean pyridinium oxidase catalysed the hydroxylation of the ring of quaternary heterocyclic compounds yielding pyridones. The oxidation of 1-methylnicotinonitrile perchlorate by pyridinium oxidase yielded 4- and 6-pyridones. The difference spectrum for this reaction was identical to the u.v. spectrum of a mixture of the known compounds.[6,12] The products of pyridinium oxidase in *Ricinus communis and Trewia nudiflora* showed similar R_f (Table 1) and u.v. spectra to the known compounds. It was of great interest to note that both 4- and 6-pyridone were also formed from the oxidation of labeled 1-^{14}C methyl nicotinonitrile iodide by *Trewia* pyridinium oxidase.[9]

TABLE 1. R_fS OF PYRIDONES

Pyridones	R_f*	U.v. detection
Authentic compounds		
2-Pyridone	0·48	Blue fluorescence
4-Pyridone	0·14	Quench
6-Pyridone	0·69	Quench
Enzymatic products from *Trewia* and *Ricinus*	0·16	Quench
	0·69	Quench

* On air-dried silica gel plates in toluene–*iso*PrOH–HOAc–H_2O (20:10:2:1).

DISCUSSION

The Euphorbiaceae consists of more than 4000 species; of 11 species tested, seven showed pyridinium oxidase activity. Of particular interest were experiments done with *Trewia nudiflora, Ricinus communis*, and *Croton tiglium. Trewia* contains an alkaloid nudiflorine (1-methyl-3 cyano-6-pyridone) which has certain structural similarity to ricinine, and is identical to a product of the castor bean (*Ricinus communis*) enzyme system. We were unable to germinate the seeds of *Croton tiglium* which have been reported to contain ricinine.[11] Extraction from the seeds also failed to show the presence of ricinine. The close resemblance in u.v. spectra of products from *Tragia involucrata* and *Ricinus communis* suggested the presence of similar enzyme systems. No attempt was made to identify alkaloids in species, other than *Ricinus* and *Trewia*, which showed oxidase activity. The question of whether there is a specific pyridone-forming enzyme in any of these species is still unresolved. In fact, aldehyde and xanthine oxidases both have been reported[13,14] to be able to oxidize a variety of pyridinium compounds. However, the importance of this study is that only plants of the Euphorbiaceae contain this pyridinium salt oxidizing enzyme.

Although Mukherjee and others[10] failed to find 4-pyridone in *Trewia*, we were able to isolate it from the crude *Trewia* enzyme incubation mixture. The absence of 4-pyridone in the earlier work could possibly have resulted from the failure of non-polar solvent extraction, since 4-pyridone is relatively polar by nature (see Table 1). Another possibility is that 4-pyridone is rapidly metabolized *in vivo*.

[13] L. GREENLEE and P. HANDLER, *J. Biol. Chem.* **239**, 1090 (1964).
[14] K. V. RAJAGOPALAN and P. HANDLER, *J. Biol. Chem.* **239**, 2027 (1964).

EXPERIMENTAL

Enzyme Preparation

The aerial portions (hypocotyl, cotyledon and endosperm) of the 5-day-old seedlings were used. The homogenate were prepared as previously described,[6] except that a 10^{-3} M EDTA was added to the buffer solution. The freshly harvested leaves were washed with distilled water and cut into 1 cm^2 pieces. These leaves (1 part by wt.) were homogenized for 1 min with 0·1 M K_2HPO_4 solution (2 parts by vol.) containing 0·001 M EDTA and 0·001 M Dithiothreitol and Polyclar AT (0·5 part by wt.). The homogenate was strained through four layers of cheesecloth and adjusted to pH 8·5 by 0·5 N KOH. From this step on, the homogenate was treated in the exact manner as described in the treatment of seedlings.

Enzyme Assay

Enzyme activity was routinely assayed in 0·05 M $NaHCO_3$ buffer containing 0·001 M EDTA at pH 9·5. Unless otherwise stated, $2{\cdot}5 \times 10^{-3}$ M 1-methylnicotinonitrile perchlorate was used as a substrate. The appearance of an enzymatic product was measured spectrophotometrically at 254 nm over a period of 30 min. The sample compartment was thermostated at 40°. Also complete difference spectra over the range of 340–220 nm were made at 10-min intervals. Activity according to Robinson[6] was defined as absorbance change in 10 min × 100.

Analysis of Enzymatic Products[9]

The incubation mixture consisted of 0·5 ml enzyme solution, 1 ml 10^{-3} M 1-methylnicotinonitrile iodide (methyl-^{14}C), 1 ml 5×10^{-3} M 1-methylnicotinonitrile perchlorate and 7·5 ml 0·05 M $NaHCO_3$ buffer, pH 9·5, with 0·001 M EDTA. It was incubated at 40° for a given time, then frozen immediately in dry ice–acetone bath, and the flask was connected to the Virtis apparatus for lyophilization. 10 ml $CHCl_3$ was then added to the dry residue, the solution was filtered through Teflon membrane (metrical pore size 0·20, 13 mm dia.) using a 10 ml syringe attached to a Swinny Adapter (Millipore Co.) and the clear filtrate was concentrated to about 0·5 ml by sweeping the solution with N_2. The concentrated filtrate was further analyzed by TLC (see Table 1) with known compounds. The 2 × 8 in. TLC plates were scanned by a strip Scanner (Nuclear-Chicago Model 1620) with attached recorder.

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