Phytochemistry, 1972, Vol 11, pp 95 to 103 Pergamon Press Printed in England

ISOLATION AND CHARACTERIZATION OF PYRIDINIUM OXIDASE B

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(*Received* 6 *April* 1971)

Abstract—Pyridinium oxidase B from *Ricinus communis*, initially separated by DEAE cellulose column chromatography, was further purified The fraction with the highest activity from Ecteola cellulose column still showed a trace of impurities in polyacrylamide gel electrophoresis Molecular weight estimation by sucrose gradient ultracentrifugation showed that pyridinium oxidase B has an apparent molecular weight of approximately 250,000 The rate of 4- and 6-pyridone formation in the catalytic process was studied The ratio of the 6-pyridone to 4-pyridone ranged from 3 5 to 4 1 The K_m for 1-methyl-nicotinonitrile perchlorate was found to be 5×10^{-4} M for pyridinium oxidase B, and it decreased with increasing pH An approximate pK_a of 9 9 was obtained for the ionizing group involved in the enzymatic reaction The K_m values for its substrate analogues, except 1-methyl-3-acetyl pyridinium, were found to be the same However, the value of V_m increased as the dipole moment of the 3 substituent increased The present evidence indicates that the positively charged quaternary nitrogen is necessary for the enzyme-substrate binding, and a strong electron withdrawing group will enhance the oxidation reaction The enzyme resembles aldehyde and xanthine oxidase in its ability to oxidize a wide variety of nitrogenous compounds

INTRODUCTION

IN THE previous paper[1] we have reported a multiple enzyme system in *Ricinus communis*. Although these three pyridinium oxidases A, B and C exhibited different behavior on DEAE-cellulose columns, suggesting that they are different protein molecules, they have many properties in common. Their substrate specificities were alike, except in the case where xanthine was used as a substrate and only pyridinium oxidase A exhibited a trace of xanthine oxidase activity. Analysis of the products also showed that all three enzymes catalysed the formation of 4- and 6-pyridones. This showed a striking similarity to enzymes such as aldehyde oxidase, xanthine and 1-methylnicotinamide oxidase from the mammalian system which is also known to have pyridone forming activities.[2–7] Comparison of the pyridinium oxidases with these mammalian enzymes, which catalyse similar reactions, is also of general interest Among these three *Ricinus* enzymes, pyridinium oxidase B is considered to be more stable and more abundant in the crude enzyme preparation. It was therefore selected for further studies.

[1] P. FU, J. KOBUS and T ROBINSON, *Phytochem* (In press)
[2] K V RAJAGOPALAN and P HANDLER, *J. Biol Chem* **239**, 2022 (1964)
[3] S CHAYKIN, *Biochim Biophys Acta* **82**, 633 (1964)
[4] L GREENLEE and P HANDLER, *J Biol Chem* **239**, 1090 (1964)
[5] K MURASHIGE, D MCDANIEL and S CHAYKIN, *Biochim Biophys Acta* **118**, 556 (1966)
[6] S GLUEKSOHU-WAELSCH, P. GREENGARD, G. P QUINN and L S TEICHER, *J Biol Chem.* **242**, 1271 (1967)
[7] R L FELSTED and S CHAYKIN, *J Biol Chem* **242**, 1274 (1967)

RESULTS

Purification

As shown in the previous paper,[1] pyridinium oxidases A, B and C were separated by DEAE-cellulose column chromatography. Pyridinium oxidase B appeared in fractions between 0 15 M and 0·25 M potassium phosphate buffer Since active enzyme fractions came out through the void volume of Sephadex G200, gels with a higher fractionation range were used The elution pattern of pyridinium oxidase B on Biogel 5A as demonstrated in Fig 1 showed that the active protein came out on the shoulder of the second major peak

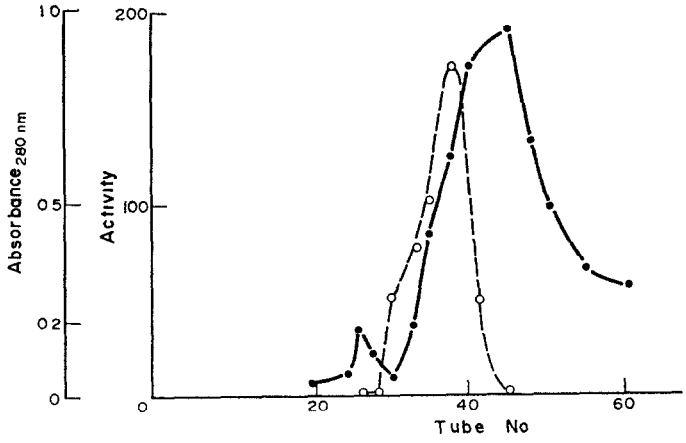

Fig 1 The elution pattern of pyridinium oxidase B on biogel 5A
The gel filtration was performed under the following conditions enzyme sample, 7 5 ml concentrated enzyme B solution from DEAE column, bed dimension, 2 5 × 50 cm, flow rate, 3 ml/20 min, eluant 0 01 M potassium phosphate, pH 7 5, 10^{-3} M EDTA, fraction volume, 3 ml/tube, operating pressure, 4 5 cm The elution was accomplished by upward flow The protein content was measured by absorbance at 280 nm The pyridinium oxidase activity was estimated as described in the Experimental
(●) Absorbance at 280 nm, (○) Activity $\left(\frac{\Delta \text{ O D } 255 \text{ nm}}{\text{ml} \times 10 \text{ min}}\right)$

Fractions collected from gel filtration were usually not homogeneous as shown by disc electrophoresis. Further purification of pyridinium oxidases B was attempted using a weak anion exchanger—Ecteola The protein as measured by absorbance at 280 nm as well as by oxidase activity are shown in Fig 2 The fraction with the highest activity was analysed by disc electrophoresis. A major fast moving band followed by a slow moving minor component was observed. Molecular weight estimation by sucrose gradient ultracentrifugation showed that the pyridinium oxidase B had an apparent molecular weight similar to that of catalase (250,000).

Analysis of Enzymatic Products

Having shown the formation of 4- and 6-pyridones by pyridinium oxidases, the ratio of 4- and 6-pyridone formation in the catalytic process was investigated The purified pyridinium oxidase B was incubated with methyl-^{14}C labelled nicotinonitrile iodide as substrate, the extraction and separation of the products were followed as described in the experimental section The above data confirm the previous nonlabelled experiment which had

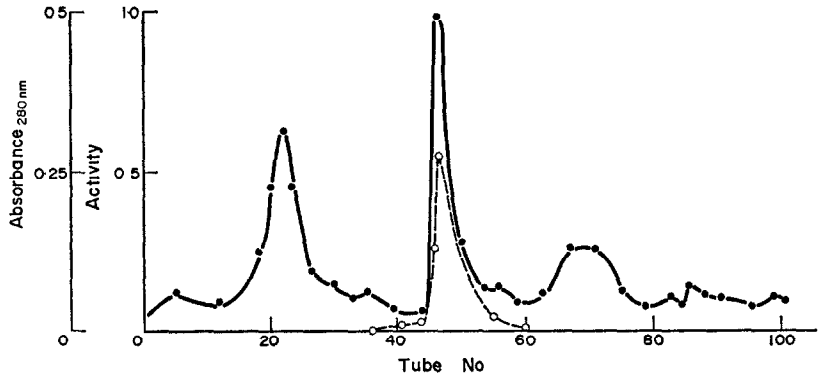

FIG 2 A TYPICAL STEPWISE ELUTION PATTERN OF PYRIDINIUM OXIDASE B FROM ECTEOLA CELLULOSE COLUMN

The Ecteola column chromatography was performed under the following conditions, enzyme sample, 20 ml enzyme B solution (12 mg) from Biogel column, bed dimension, 1 5 × 15 5 cm, flow rate, 2 ml/20 min, eluant (stepwise gradient), 0 01 M–0 08 M potassium phosphate, pH 7 5, 10^{-3} M EDTA, fraction volume, 2 ml/tube The protein content was determined by measuring absorbance at 280 nm The activity was estimated as described in Experimental

(●) Absorbance at 280 nm, (○) Activity $\left(\frac{\Delta \text{ O D } 282 \text{ nm}}{\text{ml} \times \text{min}}\right)$

shown 4- and 6-pyridones as the enzymatic products In the absence of enzyme there was less than 3% of radioactivity found in the spots of the corresponding 2-, 4- and 6-pyridones. This might have resulted either from the tailing of a solvent migration or from some nonenzymic reaction. Nevertheless, the amount of radioactivity was negligible in comparison with that found in the enzymatic reaction. It was noted in Fig 3 that the rate of

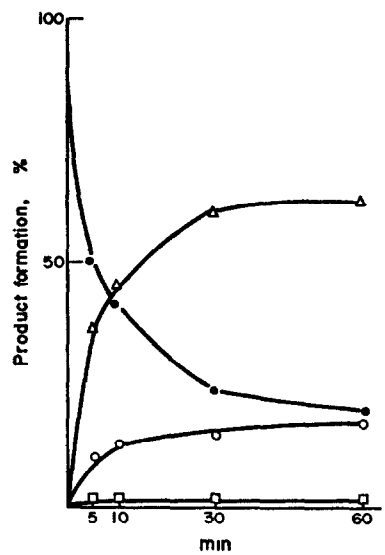

FIG. 3. THE RATE OF 4- AND 6-PYRIDONE FORMATION CATALYSED BY PYRIDINIUM OXIDASE B.
(●) Substrate, 1-methyl nicotinonitrile iodide, (□) 2-pyridone, (○) 4-pyridone; (△) 6-pyridone

4- and 6-pyridone formation leveled off after 30 min The ratio of the 6- to 4-pyridone ranged from 3·5 to 4·1 over a period of 1 hr The predominant product seemed to be 6-pyridone As shown previously,[8] several substrate analogues were oxidized by pyridinium oxidase

The UV spectra of their enzymatic oxidation products are illustrated in Fig 4 The UV spectrum of the reaction products of both 1-methyl-3-aldehyde pyridinium iodide and 1-methyl-3-acetyl pyridinium iodide were similar (Fig 4-C and 4-D) Both spectra showed maximum absorption at 280 nm The products of 1-ethyl-3-cyano pyridinium bromide and 1-benzyl-3-cyano pyridinium chloride oxidation exhibited spectrum identical to that of the oxidation product of 1-methyl-3-cyano pyridinium perchlorate See Fig 4-A, 4-F and 4-G The oxidation product of 1-methyl-3-nitro pyridinium iodide showed a maximum absorption at 300 nm (Fig 4B)

Substrate Kinetics

The effect of various concentrations of substrate analogues on the pyridinium oxidase B activity was studied (Table 1) There was no evidence of sigmoidal kinetics, and a Michaelis–Menten type kinetics was generally observed The Michaelis constant (K_m) was $5\,0 \times 10^{-4}$ M

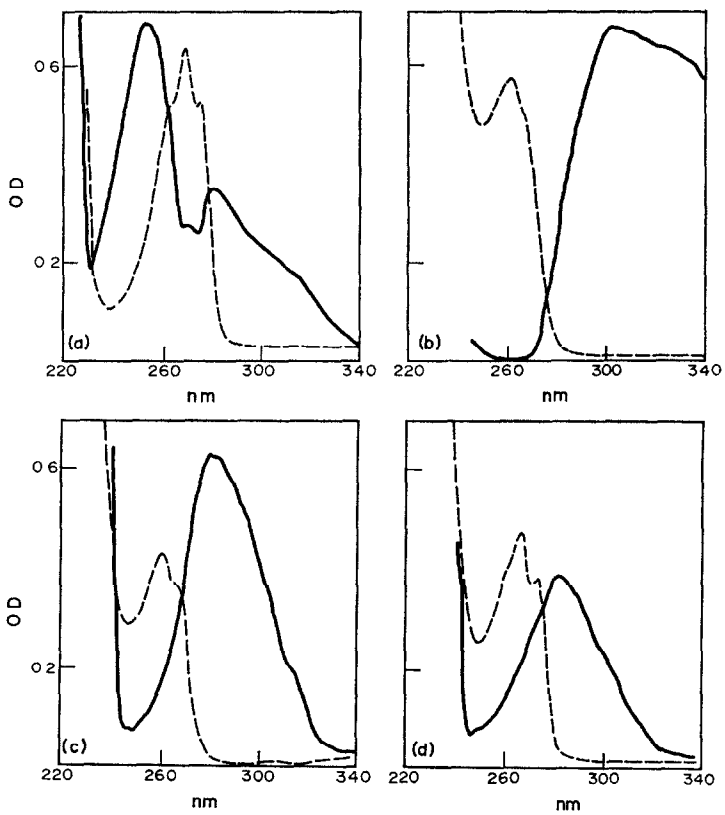

Fig 4 (a—d)

[8] P Fu, *Biochemical Studies of Pyridinium Oxidase from* Ricinus communis Ph D Dissertation University of Massachusetts (1969)

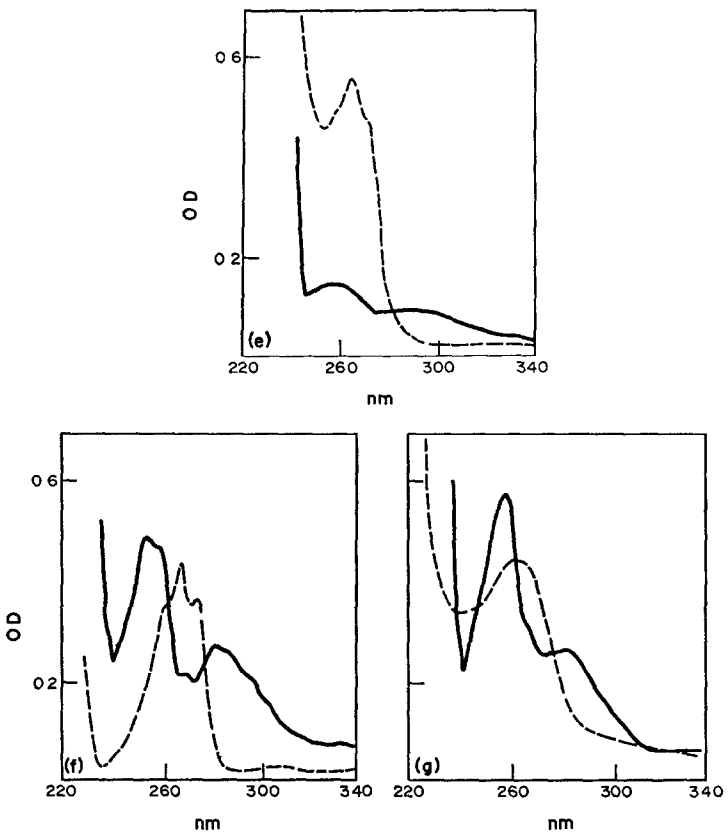

FIG. 4 (e—g)

FIG 4 UV ABSORPTION SPECTRA OF THE OXIDATION PRODUCT OF VARIOUS SUBSTRATE ANALOGUES Figure 44, Substrate (10^{-3} M) (a) 1-Methyl-3-cyano-pyridinium perchlorate, (b) 1-methyl-3-nitro-pyridinium iodide, (c) 1-methyl-3-aldehyde-pyridinium iodide, (d) 1-methyl-3-acetyl-pyridinium iodide, (e) 1-methyl nicotinamide iodide, (f) 1-ethyl-3-cyano-pyridinium bromide, (g) 1-benzyl-3-cyano-pyridinium chloride, (- - - - -) absorption spectra of substrate, (———) absorption spectra of the oxidation product

for all substrate analogues except 1-methyl-3-acetyl pyridinium iodide, and the reason for this is as yet unresolved. The V_m values illustrated the electron withdrawing potential of the 3-substituents (Table 1)

TABLE 1 K_m AND V_m VALUES FOR VARIOUS SUBSTRATES OF PYRIDINIUM OXIDASE B

Substrate	K_m (M)	V_m
1-Methyl-3-cyano-pyridinium perchlorate	5×10^{-4}	20
1-Methyl-3-nitro-pyridinium iodide	5×10^{-4}	10
1-Methyl-3-acetyl-pyridinium iodide	2×10^{-3}	6 6
1-Methyl-3-aldehyde-pyridinium iodide	5×10^{-4}	5 7
1-Ethyl-3-cyano-pyridinium bromide	5×10^{-4}	6
1-Benzyl-3-cyano-pyridinium chloride	5×10^{-4}	5

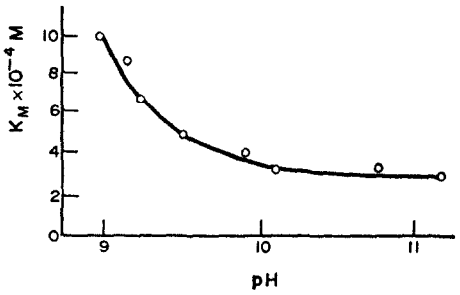

Fig. 5 The effect of pH on the K_m values for 1-methyl nicotinonitrile perchlorate as substrate for pyridinium oxidase B

Substrate analogues not oxidized by pyridinium oxidase B are trigonelline sulphate, quinoline and pyridine methiodides, nicotinic acid, nicotinonitrile and 1-methyl-3-cyano-, derivatives of 2-, 4- and 6-pyridone As shown previously,[8] the optimal activity for pyridinium oxidases A, B and C was between pH 9·5 and 10·5 The activity above pH 11 and below 9 5 fell sharply Since the quaternary compounds such as 1-methyl nicotinonitrile

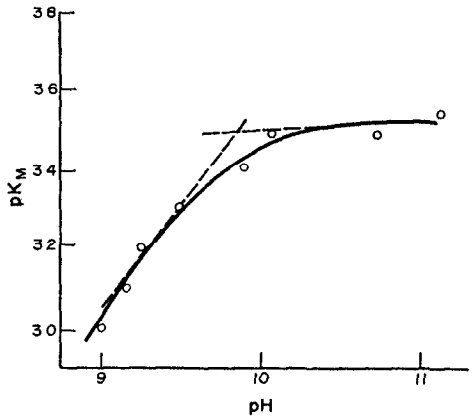

Fig 6 Dixon plot[9] of the effect of pH on K_m values for pyridinium oxidase B The intersection of the two tangents indicates the approximate pK_a of a titratable group on the enzyme

perchlorate undergo no change in ionization in the pH range of 9 0 to 11 0, the fall on either side of that optimal pH range might have resulted from the change in the affinity of an enzyme for its substrate or probably from the denaturation of the enzyme itself Thus the influence of pH on the K_m values for 1-methyl-nicotinonitrile perchlorate was further studied It was noted that the K_m values decreased as the pH was raised This is shown in Fig 5 When the data were plotted according to Dixon,[9] as shown in Fig 6, an approximate

[9] M Dixon and E. Webb, *Enzymology* (2nd Edition), Academic Press, New York (1965)

pK_a of 9·9 was obtained for the ionization group. The implication of these data will be discussed in a later section.

DISCUSSION

As described above, pyridinium oxidase B was further purified to near homogeneity. Sephadex G200, Biogel A5M and Sepharose 4B gave relatively the same resolution in purification, yet Biogel 5A and Sepharose 4B were by far easier to manipulate in column chromatography Generally, approximately 500-to 1000-fold purification was achieved after gel filtration. Individual fractions from Ecteola yielded as high as 3800-fold purification, and yet a trace of impurities was still seen in disc electrophoresis. Percentage yield of the active protein resulting from the various methods was still rather low. Therefore, in order to get sufficient purified enzyme for various biochemical studies, the present purification procedure still needs further improvement

The enzymatic oxidation products identified were 4- and 6-pyridones. The formation of 6-pyridone seems to be three to four times greater than the formation of 4-pyridone A number of partially purified 1-methyl nicotinamide oxidases from liver homogenate in the mammalian system were also shown to form 4- and 6-pyridone in various ratios [5–7,10] The importance of this enzyme in living organisms is still unclear, it is possible that it may play a regulatory role in the pyridine nucleotide cycle of NAD metabolism

In the study of substrate kinetics, similar K_m values were found for all substrate analogues except 1-methyl-3-acetyl pyridinium iodide, yet their V_m values varied in accordance with the electron withdrawing power of the 3 substituents, increasing as the dipole moment of this group increased (Table 2) The present evidence indicates that the positively charged quaternary nitrogen is necessary for the enzyme-substrate binding, while a strong electron withdrawing group at the 3 position would enhance the oxidation itself Failure to observe the oxidation of nicotinic acid and nicotinonitrile verifies the central role of the positively

TABLE 2 COMPARISON OF V_m AND DIPOLE MOMENT (D) FOR VARIOUS SUBSTRATE ANALOGUES OF PYRIDINIUM OXIDASE B

Substrate	V_m	Dipole moment (D)
1-Methyl-3-cyano-pyridinium perchlorate	20	4 39
1-Methyl-3-nitro-pyridinium iodide	10	4 21
1-Methyl-3-acetyl-pyridinium iodide	6 6	3 00
1-Methyl-3-aldehyde-pyridinium iodide	5 7	2 76

charged quaternary nitrogen in the enzymatic mechanism. It was noted that trigonelline was not oxidized by pyridinium oxidase, the negatively charged carboxyl group possibly being repelled from the binding site. As shown in Fig 5 the influence of pH on K_m values for 1-methylnicotinonitrile perchlorate was striking Since the quaternary nitrogen compound is not likely to change its ionization in the pH range from 9·0 to 11 0, the observed gradual decrease in K_m values as pH increased suggested that the enzyme underwent certain structural changes at the substrate binding site If the enzyme is only active in one ionic form at the

[10] S D HUFF and S CHAYKIN, *J Biol Chem* **242**, 1265 (1967).

active site, then loss of a proton from this site could result in a higher affinity for the positively charged quaternary heterocyclic compounds From the Dixon plot, an approximate pK_a of 9·9 obtained for the ionization suggested that chemical groups like amino, sulfhydryl and phenolic hydroxyl groups would probably be involved in the catalytic process Although a number of sulfhydryl reagents exhibited inhibition on pyridinium oxidase B, the possibility of reaction with groups other than –SH cannot be excluded In considering the involvement of an amino group at or near the active site, Greenlee and Handler[4] postulated that protonation of an amino group would prevent the binding of quaternary compounds by electrostatic repulsion, yet dissociation of this group with increasing pH would allow the quaternary heterocyclic compounds to approach the binding site and be oxidized This idea could very well be applied to a negatively charged phenoxide group which in turn would attract a positively charged quaternary ammonium compound In this paper, some of the biochemical properties of pyridinium oxidase B are presented and discussed It must be concluded that much work will be required to elucidate the mechanism of action of the pyridinium oxidase

EXPERIMENTAL

Purification of Pyridinium Oxidase B

Preparation of crude enzyme solution and its separation into pyridinium oxidases A, B and C by DEAE cellulose column were described previously [1] All fractions of pyridinium oxidase B from gel filtration were pooled and concentrated by ammonium sulfate precipitation The precipitate collected by centrifugation at 12,000 rev/min for 20 min was dissolved in a minimal volume of an appropriate buffer containing 0 001 M EDTA and dialysed exhaustively

Ecteola column chromatography Ecteola (mixed amines) cellulose was first washed in alkali and then in acid 20 ml of pyridinium oxidase B solution (approximately 12 mg protein) was applied to the column (1 5 × 15 5 cm) which was previously equilibrated with 0 01 M potassium phosphate containing 0 001 M EDTA at pH 7 5 The column was eluted with a stepwise gradient from 0 01 to 0 08 M and the protein profile was monitored at 280 nm

Acrylamide gel electrophoresis The procedure of Ornstein and Davis[11] was followed, except that the 'sample' as well as 'spacer' gels were omitted, and the sample in 20% sucrose was delivered by a 50 μl Hamilton syringe directly on top of the separating gel Coomassie blue was used to detect the protein zones [12] Electrophoretic conditions were as follows 2 mA/tube, 0 05 M Tris-glycine buffer at pH 8 9, 45 min to 1 hr duration

Sucrose gradient ultracentrifuge Sucrose gradients were used in a manner similar to that reported by Martin and Ames [13] Continuous gradients of 8 5–20% sucrose in 0 05 M potassium phosphate buffer (pH 7 5) and 0 001 M EDTA were run in a Beckman L2-65B ultracentrifuge with a SW41 head with total volume of 13 ml of sucrose Approximately 500 μg of concentrated pyridinium oxidase B plus marker protein (catalase) in a volume less than 0 3 ml was layered on the top of the gradient Gradients were centrifuged at approximately 40,000 rev/min for 4 hr at 20° The gradient was tapped with a 21 G needle and 40 drop aliquots (about 0 5 ml) were collected The fractions were assayed for enzyme activity Molecular weights were calculated according to Martin and Ames [13]

Analysis of Enzymatic Products

The extraction and separation of the oxidation products was carried out as described in the previous paper [14] The characterization of the products was achieved by TLC on silica gel The chromatogram was examined under UV light and treated with Dragendorff's reagent [15] Spots that exhibited fluorescence or

[11] L Ornstein and B J Davis, *Ann N Y Acad Sci* **121**, 321 (1964)
[12] A Chrambach, *Anal Biochem* **20**, 150 (1967)
[13] R G Martin and B N Ames, *J Biol Chem* **236**, 1372 (1961)
[14] P Fu and T Robinson, *Phytochem* **9**, 2443 (1970)
[15] H M Bregoff, E Roberts and C C Delwiche, *J Biol Chem* **205**, 565 (1953)

quenching were outlined, their R_f values calculated, and they were then removed and dissolved in various solvents Filtrates from Teflon membranes were examined by UV absorption over the range of 200 to 340 nm The ratio of 4- and 6-pyridone formation in the catalytic process was investigated by using methyl-^{14}C labelled 1-methyl-nicotinonitrile iodide as substrate The extraction and separation of the products was followed as previously described Spots of 2, 4 and 6-pyridones from experimental samples as well as from controls were transferred to a scintillation counting vial, and the radioactivity counted by scintillation

Enzyme assay Chemicals as well as assay methods were as described previously [1,11]

Key Word Index—Ricinus communis, Euphorbiaceae, pyridinium oxidase B, enzyme purification