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ENZYMATIC TRANSFORMATIONS OF MORPHINANE ALKALOIDS

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Key Word Index—*Papaver somniferum*; Papaveraceae; morphine; codeine; thebaine; morphinane *N*-oxides; pseudomorphine; peroxidase; poppy enzyme fraction; phenolcarboxylic acids; ascorbic acid.

Abstract—Horseradish peroxidase transforms morphinane alkaloids into *N*-oxides and morphine to pseudomorphine in the presence of hydrogen peroxide. The crude poppy enzyme fraction shows the same activities. The rates of reactions were influenced by phenolic compounds and their relation controlled by the concentration of hydrogen peroxide and the presence of ascorbic acid.

INTRODUCTION

It has been shown that the morphine level decreases in poppy latex in the presence of oxygen, probably due to the activity of enzymes [1]. Phenolase (catechol oxidase, EC 1.10.3.1 and/or monophenol monooxygenase, EC 1.14.18.1) activity was found in seedlings of poppy [2] and active enzyme fractions were isolated from poppy plants [3] and latex [4]. Morphine is oxidized by phenolase to pseudomorphine in the presence of phenolcarboxylic acids [3]. Horseradish peroxidase (HRP; EC 1.11.1.7) and hydrogen peroxide also form pseudomorphine [5, 6], but no peroxidase activity was detectable with common donors either in the poppy plant [3] or the latex [7], despite numerous experiments. The transformation of morphine by poppy enzyme fraction in the presence of hydrogen peroxide, on which no report can be found in the literature, was one of the aims of our investigations.

More recent evidence for morphine turnover in plant suggests that morphine undergoes *N*-demethylation to normorphine [8]. *N*-Oxides readily form noralkaloids and may, therefore, be involved in *N*-dealkylation processes [9]. *N*-Oxides of morphinane alkaloids are plant products. Both *N*-oxides of morphine (isomers at the assymetric nitrogen centre) as well as one of codeine have been isolated from poppy plants; both *N*-oxide isomers of thebaine have also been obtained from a related species, *Papaver bracteatum* [10]. *In vitro* conversion of morphine to its *N*-oxides in poppy latex at a rate of 1–3% was also detected [11]. *N*-Oxides of morphinane alkaloids are generally synthesized with hydrogen peroxide [10]. Their enzymatic formation has not been investigated.

We assumed the possibility of a common peroxidative formation of pseudomorphine and *N*-oxides of morphinane alkaloids and the determining role of cofactors in the rate of both alternative reactions. A typical peroxidase, HRP, and a crude enzyme from poppy seedling were used in these reactions in the presence of hydrogen peroxide and the formation of *N*-oxides and/or of pseudomorphine directed by several cofactors and reaction parameters was observed.

RESULTS AND DISCUSSION

The oxidation of morphinane alkaloids was performed at pH 7 at room temperature. The reaction was stopped after 1 hr. The simplest system contained 4×10^{-10} M HRP, 4×10^{-6} M alkaloid and 4×10^{-5} M H_2O_2. The formation of considerable amounts (1–2%) of *N*-oxides of morphine, codeine and thebaine were detected by TLC. The same compounds were synthesized for identification. The chromatograms were developed in several solvents and the compounds were detected with different reagents (see Experimental). The enzymatically formed *N*-oxides were completely identical with the synthetic products. The amount of *N*-oxide was determined semi-quantitatively by comparison of the intensities of alkaloid spots.

Under certain conditions a dimer of morphine (i.e. pseudomorphine) was also formed. It was separated from the reaction mixture by extraction (see Experimental) and compared with synthetic materials [13] by mass, NMR and UV spectrometry and by TLC. The enzymatically formed pseudomorphine showed positive reactions with both alkaloid and phenol reagents [14]. The mass spectrum shows that the MW of the product is twice that of morphine. By NMR spectrometry it was detected that the binding between morphine molecules is in the 1, 1'- or 2, 2'-positions. The dimerization of morphine is an *ortho–ortho* coupling reaction by a free radical mechanism (the *para* position is blocked) and the pseudomorphine formed is a 2, 2'-dimer.

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The reaction was carried out at several pHs and *N*-oxide formation was detected at pH 7 and 8 but not at pH 5 and 6, so that only the basic form of the alkaloid is able to produce *N*-oxides.

Phenols of natural origin as well as ascorbic and dihydroxyfumaric acids act under certain conditions as cofactors of peroxidase [3, 12]. We observed an increased formation of *N*-oxides (up to 2–3%) caused by 4×10^{-6} M ascorbic acid. The addition of 4×10^{-7} M phenolic compounds strengthened this effect (*p*-coumaric or caffeic acid up to 4–5%, ferulic acid, 3,4-dihydroxyphenylacetic acid or DOPA up to 3–4%). Using dihydroxyfumaric acid instead of phenols no *N*-oxide formation was detected. In control experiments without native or with boiled enzyme no reaction was recorded.

The rate of reaction is controlled by the presence or absence of ascorbic acid and by the concentration of hydrogen peroxide. In the presence of ascorbic acid morphine, codeine and thebaine are oxidized by HRP, in the reaction mixture containing phenolic compound and 4×10^{-5} M hydrogen peroxide, predominantly into the corresponding alkaloid *N*-oxide isomers. The optimal molar ratio of enzyme–alkaloid–ascorbic acid–phenolcarboxylic acid–hydrogen peroxide in the reaction mixture was $1 \times 10^{-4} : 1 : 1 : 1 \times 10^{-1} : 10$. In the absence of ascorbic acid and with ten times less hydrogen peroxide, pseudomorphine is formed from morphine in the larger quantities (up to 8–10%) with some *N*-oxides (0.5%). The optimal amounts of the components in the reaction forming pseudomorphine were HRP 4×10^{-10} M, morphine 4×10^{-6} M, phenolcarboxylic acid 4×10^{-7} M, hydrogen peroxide 1.3×10^{-6} M; i.e. with the molar ratio of enzyme–morphine–phenolcarboxylic acid–hydrogen peroxide of $1 \times 10^{-4} : 1 : 1 : 1 \times 10^{-1} : 3 \times 10^{-1}$. The quantity of hydrogen peroxide used in synthetic reactions was more than 77 and 230 times, respectively, greater than in the optimal enzymatic reactions. In the absence of hydrogen peroxide the reactions were not detectable and smaller quantities of compounds were formed when only one-third or one-tenth of the optimal amounts were added.

The effect of the structure of phenolic compounds on the reaction was also examined. The largest amount of pseudomorphine (8–10%) was formed in the presence of *p*-coumaric, *p*-hydroxyphenylpyruvic or ferulic acids. We detected smaller quantities (4–5%) in the presence of 3,4-dihydroxyphenylacetic, *o*- and *m*-coumaric acids or DOPA. No pseudomorphine was formed in the presence of 3,4-dihydroxybenzaldehyde. These results show that not only the presence of a free phenolic hydroxyl group, but also the ability to form radicals, is necessary for the reaction to occur.

The reaction mixture in which pseudomorphine was detected, has a yellow colour. It is known that the stable radical of morphine prepared with $SbCl_5$ is yellow [15]. This supports the assumption that the enzymic reaction proceeds by a free radical mechanism. No dimeric alkaloids were formed from codeine or thebaine and no yellow colour appeared. Thus dimerization depends on the presence of a phenolic hydroxyl group in the alkaloid. The reaction time for dimerization was very short as compared with the formation of *N*-oxides which took some minutes. Pseudomorphine formation was detectable in a few seconds by TLC.

All the above reactions were carried out with a crude poppy enzyme fraction. This enzyme preparation contained all the soluble protein extracted by buffers from an acetone powder which precipitated with ammonium sulphate to 80% saturation, but was free of alkaloids, phenolic compounds, amines and amino acids. Poppy enzyme has negligible peroxidase activity with common donors (i.e. *o*-dianisidine, DOPA) in accordance with previously cited results [3, 7]. In spite of this, the crude poppy enzyme showed a peroxidase-like activity in the transformation of morphinane alkaloids in the presence of hydrogen peroxide to *N*-oxides and in that of morphine to pseudomorphine in the same way as HRP. On the other hand, crude poppy enzyme has a high phenolase activity, but in the absence of hydrogen peroxide neither *N*-oxides nor pseudomorphine were formed. These unexpected results suggest the need to further investigate the oxidases of poppy. The *in vitro* formation of pseudomorphine by crude poppy enzyme suggests that this alkaloid isolated from opium [16] may be not an artefact, but a natural product. Thus it may be concluded that the formation of morphine *N*-oxides and pseudomorphine are connected alternative enzymatic processes showing an analogy with the non-enzymatic transformation of morphine into *N*-oxides and pseudomorphine in aqueous solution [17]. The direction of the reaction is controlled by the concentration of hydrogen peroxide and by the presence of ascorbic acid (Table 1).

For the enzymatic oxidation of morphine we propose the following mechanism (Fig. 1). Route a: in the presence of hydrogen peroxide the enzyme transforms the phenolcarboxylic acids into free radicals and the latter generate morphine radicals dimerizing to pseudomorphine. Route b: the presence of ascorbic acid inhibits the first step and morphine *N*-oxides without any pseudomorphine are formed. This is in accord with the fact that ascorbic acid inhibits the oxidation of *p*-coumaric acid by hydrogen peroxide and peroxidase [18].

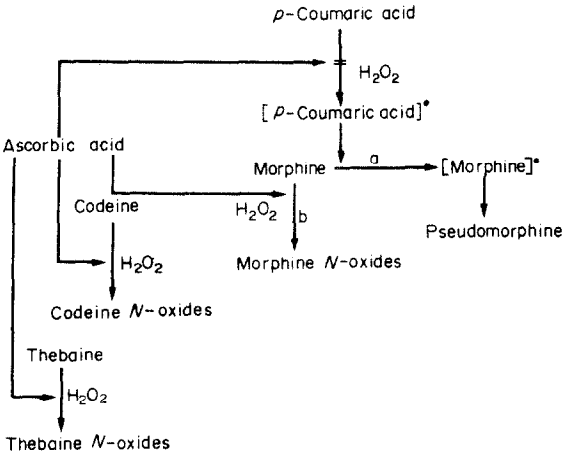

Fig. 1. Proposed pathways of enzymatic oxidation of morphinane alkaloids.

Table 1. Enzymatic oxidative transformations of morphine into its *N*-oxides and pseudomorphine in the simplest reaction mixtures

Components					
Enzyme*	+	+	+	+	+
4×10^{-5} M H_2O_2	+	+	+	−	−
1.3×10^{-6} M H_2O_2	−	−	−	+	−
1.3×10^{-7} M H_2O_2	−	−	−	−	+
4×10^{-6} M morphine	+	+	+	+	+
4×10^{-7} M *p*-coumaric acid	−	−	+	+	+
4×10^{-6} M ascorbic acid	−	+	+	−	−
Products†					
Morphine *N*-oxides	1–2%	2–3%	4–5%	0.5%	tr‡
Pseudomorphine	−	−	−	8–10%	2–3%

*4×10^{-10} M HRP or 0.5 mg crude poppy enzyme.
†100% is 4×10^{-6} M morphine.
‡Traces (0.1–0.2%).

EXPERIMENTAL

Synthetic preparations. Alkaloid *N*-oxides were prepared as described by Phillipson et al. [9] and pseudomorphine by the method of Bentley and Dyke [13].

Enzymatic transformation of alkaloids to N*-oxides.* The reaction mixture contained: 1 mg alkaloid (morphine, thebaine, codeine) in 1 ml Pi buffer, pH 7; 0.05 mg phenolic compound in 0.1 ml H_2O; 0.1 ml 0.1% H_2O_2; 1.4 mg ascorbic acid in 0.2 ml H_2O and 0.025 mg HRP (or 0.5 mg poppy enzyme) in 0.1 ml phosphate buffer. The reaction was started by addition of enzyme and stopped by adding 0.8 ml EtOH and boiling. The mixture was evaporated *in vacuo*. The dry residue was dissolved in 1 ml EtOH and identified by TLC.

Enzymatic transformation of morphine to pseudomorphine. The same as that for the *N*-oxides, but the reaction mixture did not contain ascorbic acid.

Enzymatic preparation of pseudomorphine. To obtain larger amounts of product, 200 times greater quantities of compounds were used in the reaction mixture.

200 mg morphine was dissolved in 400 ml Pi buffer (pH 7); 10 mg *p*-coumaric acid in 20 ml H_2O; 20 ml 0.1% H_2O_2 and 5 mg HRP in 20 ml H_2O was added. After 60 min. 280 ml EtOH was added, the reaction was stopped by boiling and evaporated *in vacuo*. The dry residue was dissolved in 10 ml H_2O and extracted with 3×20 ml CH_2Cl_2 at pH 10 and the same was done at pH 11. The pH of the aq. phase was brought to 9 and extracted with $CHCl_3$–*iso*-PrOH (3:1) (5×20 ml). This fraction was evaporated *in vacuo*. The dried fraction was dissolved in 50% EtOH and pseudomorphine was crystallized. The remaining pseudomorphine was ppted by conc. HCl from the mother liquor.

TLC identification of reaction products. Alkaloid *N*-oxides were identified in four solvent systems [9]. Separation of pseudomorphine and morphine was carried out in Me_2CO–H_2O–NH_4OH (80:15:4.5), using Si gel GF_{254} (Merck) with R_f values 0.60 and 0.68 respectively. The spots were located in UV light, by detection with Dragendorff's and potassium iodoplatinate alkaloid reagents. Morphine and its *N*-oxides as well as pseudomorphine reacted with Folin–Ciocalteu and the $FeCl_3$–$K_3Fe(CN)_6$ phenolic reagents [14].

Spectral data of pseudomorphine. UV spectra in pH 9 Pi buffer: pseudomorphine $\lambda_{(1)max} = 239$ nm, $\lambda_{(2)max} = 280$ nm (morphine $\lambda_{(1)max} = 214$ nm, $\lambda_{(2)max} = 290$ nm). MS data: 70 eV, 568 $[M]^+$ (100), (M = 568.2612; $C_{34}H_{36}N_2O_6$), 285 [M/2] (20), 550 $[M-18]^+$ (20), 275 $[550]^{2+}$ (20). NMR data: ^1H NMR (100 MHz, DMSO-d_6-CDCl$_3$, 1:1); pseudomorphine: 6.38 (1H, *s*, H-1), 5.62 (1H, *m*, $J_{7\alpha,8\alpha} = 9.6$ Hz, H-7), 5.28 (1H, *m*, $J_{8\alpha,7\alpha} = 9.6$ Hz, H-8), 4.79 (1H, *dd*, $J_{5\alpha,6\alpha} = 6.8$ Hz, $J_{5\alpha,7\alpha} = 1.4$ Hz, H-5), 4.12 (1H, *m*, H-6), 2.37 (3H, *s*, N-17).

Isolation of crude poppy enzyme. 2-Week-old poppy seedlings were homogenized at 0–5° in the presence of $Na_2S_2O_3$ and Polyclar AT in Me_2CO. After filtration the homogenization was repeated ×15. The dried Me_2CO powder was homogenized ×3 in borate buffer (pH 7.6) and centrifuged 18 min at 12000 *g*. The proteins were ppted from the supernatant with $(NH_4)_2SO_4$ to 80% satn. The ppt was dissolved in borate buffer (pH = 7.6) and dialysed. The soln containing soluble proteins was freeze-dried and used as crude poppy enzyme.

Peroxidase activity of crude poppy enzyme. 0.05 ml 1% *o*-dianizidine in H_2O + 6 ml 0.003% H_2O_2 in Pi buffer (pH 6); 2.9 ml of this mixture was put in a 1 cm^3 cuvette, the remaining soln in the blank control. The reaction was started with 0.1 g enzyme in 0.1 ml H_2O. The poppy enzyme showed 1000 times lower activity at 460 nm than HRP with 500 units/mg from commercial source.

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REFERENCES

1. Nilov, V. I., Nilova, V. I. and Trotschenko, A. T. (1963) *Biochimija* **1**, 165.
2. Jindra, A. (1967) *Acta Fac. Pharm. Bohemoslov.* **13**, 7.
3. Schenck, G., Froemming, K. H., Wiechula, W. and Schwalb, E. (1960) *Arch. Pharm.* **293**, 312.
4. Roberts, M. F. (1971) *Phytochemistry* **10**, 3021.
5. Schenck, G., Froemming, K. H. and Schneller, H. G. (1965) *Arch. Pharm.* **298**, 855.
6. Roerig, D. L., Reak, C. J. and Wang, E. H. L. (1976) *Biochem. Pharmacol.* **25**, 1075.
7. Antoun, M. D. and Roberts, M. F. (1975) *Phytochemistry* **14**, 909.
8. Miller, R. J., Jollers, C. and Rapoport, H. (1973) *Phytochemistry* **12**, 597.

9. Phillipson, J. D. and Handa, S. S. (1978) *Lloydia* **41**, 385.
10. Phillipson, J. D., Handa, S. S. and El-Dabbas, S. W. (1976) *Phytochemistry* **15**, 1297.
11. Fairbairn, J. W., Handa, S. S., Gürkan, E. and Phillipson, J. D. (1978) *Phytochemistry* **17**, 261.
12. Gibson, Q. H. (1968) in *Biological Oxidations* (Singer, P. T., ed.), pp. 379–413. Interscience, New York.
13. Bentley, K. W. and Dyke, S. F. (1959) *J. Chem. Soc. C* 2574.
14. Kirchner, J. G. (1978) *Thin Layer Chromatography* pp. 193–264. J. Wiley, New York.
15. Leterrier, F. and Viossat, B. (1968) *C.R. Acad. Sci. Ser. C* **265**, 410.
16. Boit, H. G. (1961) *Ergebnisse der Alkaloid-Chemie bis* 1960. Akademie, Berlin.
17. Yeh, S. Y. and Lach, J. L. (1961) *J. Pharm. Sci.* **50**, 35.
18. Stafford, H. A. and Baldy, R. (1970) *Plant Physiol.* **45**, 215.