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CONVERSION OF CODEINE TO MORPHINE IN ISOLATED CAPSULES OF *PAPAVER SOMNIFERUM*

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Key Word Index—*Papaver somniferum*; isolated capsules; biotransformation; codeine; morphine; cofactors; codeine *N*-oxide; morphine *N*-oxide.

Abstract—The biotransformation of codeine to morphine was studied in isolated capsules of *Papaver somniferum*. Cofactors such as nicotinamide adenine dinucleotide, adenosine 5′-triphosphate, *S*-acetyl coenzyme A and pyridoxal phosphate were not required in the conversion of codeine to morphine. Reducing agents such as dithiothreitol, glutathione and β-mercaptoethanol strongly promoted codeine and morphine degradation, while morphine formation remained at a constant level. Hydrogen peroxide (concentration > 0.25 mM) caused the conversion of codeine and morphine to *N*-oxides by non-enzymatic oxidation. Isolated capsules of *P. somniferum* provide a method of studying the biotransformation of codeine to morphine.

INTRODUCTION

During the last two decades, the enzymatic biosynthesis of the morphine alkaloids has been studied [1–3]. $^{14}CO_2$ exposure and radioactive labeled precursor feeding experiments with intact *Papaver somniferum* plants [1–5] were the tools employed to demonstrate the biotransformation of morphine alkaloids. There were indications that following its formation morphine was degraded to non-alkaloid metabolites [6, 7]. In the biosynthetic pathway, Hodges and Rapoport [8] demonstrated the conversion of codeinone to codeine by cell-free extracts of the entire plant of *P. somniferum*. With the same cell extract, Hodges and Rapoport [9] reported a high yield conversion of [^{3}H]reticuline to [^{3}H]salutaridine. Neither cell-free extracts nor latex have been used to study the conversion of codeine to morphine. In this study, we used isolated capsules of *P. somniferum* to study this important step in the biotransformation. The objective of this investigation was to determine the role of enzyme cofactors and other factors in controlling the conversion of codeine to morphine.

RESULTS AND DISCUSSION

[^{14}C]*Codeine uptake and metabolism in capsules*

One week after petal fall, young capsules of *P. somniferum* were excised and incubated with [^{14}C]codeine in MS medium [10]. The uptake of radioactivity into the capsules increased with increasing incubation time (Fig. 1). Uptake reached near maximum at 10 hr; at that time, the amount of radioactivity in the capsules was only 15% of the [^{14}C]codeine initially added to the medium.

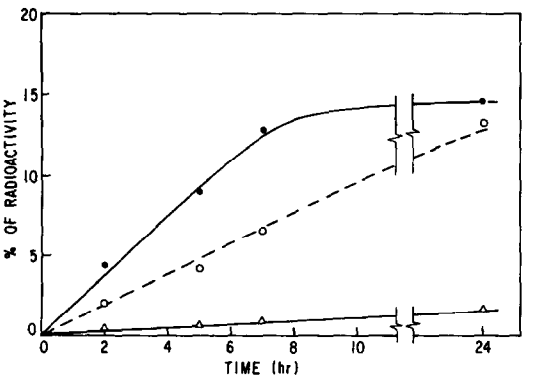

Fig. 1. [^{14}C]Codeine uptake into excised capsules. Excised capsules were incubated with [^{14}C]codeine in MS medium and harvested at various times as described in the Experimental. The uptake of radioactivity by capsules (●——●) was calculated by subtracting the amount of radioactivity left in the medium after the incubation from that of the initial [^{14}C]codeine added to the medium after chloroform–isopropanol extraction. The two fractions that were separated: (○- - -○) the chloroform–isopropanol extractable fraction, and (△——△) the aqueous fraction.

The [^{14}C]codeine that was taken up was metabolized into two fractions; the chloroform–isopropanol extractable fraction contained the alkaloid products and the aqueous fraction contained the non-alkaloid degradation products. Radioactivity in both the chloroform–isopropanol and aqueous extracts increased with the time of incubation. The aqueous extract contained less than 10% of the radioactivity that was taken up by the capsules and a detailed analysis of these products was not attempted. The chloroform–isopropanol extract was separated by TLC and the plates were analysed by TLC scanning, which indicated that radioactive morphine, morphine *N*-oxide, codeine *N*-oxide, trace amounts of normorphine, and other unidentified products were present in the extract. These findings suggested that the capsules contained

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demethylase and oxidase enzyme(s) capable of converting [^{14}C]codeine to morphine and its *N*-oxides, respectively. Conversely, at zero time of incubation, the control experiment showed that no [^{14}C]codeine was present in the capsules, and the remainder of [^{14}C]codeine in the medium was not metabolized to the other products described above. The detailed physical and chemical evidence will be discussed later. One of the important ^{14}C-labeled products was identified as morphine by TLC mobility comparisons. Further proof of the identity of radioactive morphine was obtained by comparing the specific activity of [^{14}C]morphine. [^{14}C]Morphine isolated by preparative TLC was passed through a HPLC column and recovered. The recovered [^{14}C]morphine was reinjected onto the HPLC column. After repeating three times, the specific activity (total cpm/mg) of [^{14}C]morphine remained nearly constant in two independent experiments. These data substantiate that the [^{14}C]morphine was chemically identical to non-radioactive morphine.

Cofactor involvement in the conversion of [^{14}C]*codeine to* [^{14}C]*morphine*

Possible cofactors such as pyridoxal phosphate, nicotinamide adenine dinucleotide (NAD), adenosine 5′-triphosphate (ATP), *S*-acetyl CoA, or a divalent cation (Mg^{2+}), reducing agents, or hydrogen peroxide (H_2O_2) were individually included in the incubation mixture (Table 1). Except for glutathione and H_2O_2, other cofactors and Mg^{2+} did not affect the transformation of [^{14}C]codeine to [^{14}C]morphine; the percentage of products (morphine and *N*-oxides) remained almost constant. High concentrations of cofactors gave only slightly different results. At 2 mM pyridoxal phosphate, the formation of morphine was increased slightly (10–20%), while *N*-oxides remained unchanged. Several concentrations of H_2O_2 were tested to ascertain whether the mechanism of the effect of H_2O_2 was through enzymatic catalysis or non-enzymatic oxidation. Different concentrations of H_2O_2 (0.025, 0.25, 0.5 mM) were incubated in medium containing [^{14}C]codeine with or without capsules. In the controls (without capsules), when H_2O_2 concentrations were greater than 0.25 mM, [^{14}C]codeine was oxidized non-enzymatically to the *N*-oxide. At a low concentration of H_2O_2 (0.025 mM), both incubations (with or without capsules) did not change the conversion. The above results suggest that low concentrations of H_2O_2 did not affect the conversion of [^{14}C]codeine to [^{14}C]morphine, while higher concentrations of H_2O_2 (> 0.25 mM) caused *N*-oxide formation via non-enzymatic reaction. At the same concentration of H_2O_2 (0.025 mM), Vagujfari and Petz-Stifter [11] have reported that crude poppy enzyme fraction was able to transform morphinane alkaloids into *N*-oxides and morphine to pseudo-morphine. Several reducing agents such as dithiothreitol (DTT), glutathione and β-mercaptoethanol at various concentrations were incubated with excised capsules in the presence of [^{14}C]codeine (Fig. 2). Results obtained by TLC separation and scanning indicated that when reducing agents were added, the formation of *N*-oxides was stimulated. At 1 mM concentration, β-mercaptoethanol and glutathione were more effective than DTT. The amount of [^{14}C]codeine was much smaller than the control, while the amount of morphine was not significantly changed (Table 1). The effect of reducing agents may be (1) [^{14}C]codeine oxidation to the *N*-oxide was enhanced, and/or (2) the rate of formation of [^{14}C]morphine from [^{14}C]codeine and the rate of morphine oxidation were both enhanced. Reducing agents have been found to inhibit oxidases [12]. The study presented here suggests that reducing agents probably did not act directly on the enzyme itself, but served as enzyme stabilizers. A similar observation, that K^+-stimulated ATPase activity and cytochrome *c* oxidoreductase were maintained in the presence of reducing agents, has been reported in corn root [13].

Purification and identification of N*-oxide products*

As indicated above, other than [^{14}C]morphine, two additional radioactive peaks migrated a short distance from the origin. These two radioactive peaks were rechromatographed on TLC plates in a second solvent system of acetone–water–ammonium hydroxide (80:15:5, v/v). Two peaks were completely separated. On comparison with the mobility of the authentic alkaloids, these two unknown compounds appeared to be morphine *N*-oxide and codeine *N*-oxide, respectively. Further identification of these products was achieved by mass

Table 1. The effect of cofactors and other compounds on the conversion of [^{14}C]codeine to [^{14}C]morphine

		% of alkaloids*		
Cofactors	Concn (mM)	*N*-Oxides	Morphine	Codeine
1. None	—	22	21	55
2. Pyridoxal phosphate	1.0	27	22	51
3 NAD	0 5	23	22	55
4. ATP	1 0	25	21	53
5. Glutathione	1.0	55	18	27
6. $MgCl_2$	1.0	23	18	59
7. *S*-Acetyl CoA	0.5	20	56	
8. H_2O_2	0.5	63	5	35
9. Mix†	—	63	14	23

*The percentage of alkaloids was obtained from TLC scanning.

†The incubation contained all the chemicals in this table at the concentration described above.

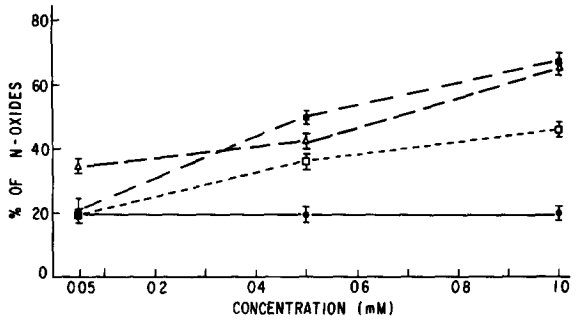

Fig. 2. The effect of various concentrations of reducing agents on the conversion of [^{14}C]codeine to [^{14}C]morphine. Various reducing agents: control (●——●), DTT (□- - - □), glutathione (△- - -△), and β-mercaptoethanol (■· — · —■) were incubated with excised capsules as described in the Experimental. After TLC scanning, the percentage of *N*-oxide formation (total integrated area of combined two peaks located near the origin of excised capsules at zero time incubation) was plotted against the concentrations of various reducing agents.

Table 2. Mass spectrometric characterization of the products from the conversion of [^{14}C]codeine to [^{14}C]morphine in isolated capsules of *Papaver somniferum*

Compounds*	Relative abundance of major ions (*m/z*)†	
	70 eV	NH_3 CI
Morphine (MW 285)	285, 268, 215, 174, 162, 124	286, 303
Morphine *N*-oxide (MW 301)	301, 285, 268, 215, 174, 162, 124	303, 286
Codeine *N*-oxide (MW 315)	298, 241, 299, 315, 225	282, 300, 316

*These compounds were purified by TLC. Thermal decomposition in the vaporization process in the mass spectrometer was not monitored.

†The intensity is expressed as descending order.

spectrometry. Table 2 shows the mass spectral data for the three compounds that were isolated from the incubation in comparison with the authentic compounds. Both EI and CI (NH_3) mass spectrometric methods conclusively identify the incubation products as morphine, morphine *N*-oxide and codeine *N*-oxide. The mass spectral identifications were consistent with the results obtained from the TLC mobilities.

The isolated capsule system described here provides an ideal means for studying the biotransformation of morphine alkaloids in *P. somniferum*. The advantages of the system include: (1) it is close to the *in vivo*, whole plant system; (2) it does not denature the biosynthetic enzyme system; and (3) it provides uniform precursor uptake.

The biotransformation of codeine to morphine is not a simple one substrate to one product reaction. Several possible reactions have to be considered (Fig. 3). In this study, [^{14}C]codeine and [^{14}C]morphine were converted to their radioactive *N*-oxides. Latex or whole plants of *P. somniferum* have the ability to convert morphine and codeine into their *N*-oxides [14, 15]. Demethylation of morphine in *P. somniferum* has been established as an active metabolic process [16]. Normorphine was found in

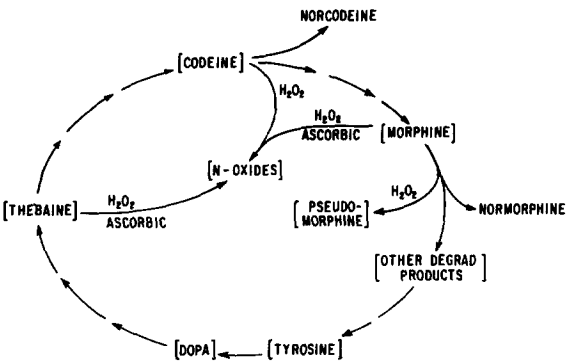

Fig. 3. Proposed pathway of conversion of codeine to morphine.

raw opium [15]. The morphine degradative pathway involves an initial demethylation to normorphine, which is subsequently degraded to non-alkaloid metabolites. Morphine alkaloids may play an active metabolic role, perhaps as specific methylating agents. In the presence of H_2O_2, horseradish peroxidase transformed alkaloids into *N*-oxides and morphine to pseudo-morphine [11]. The crude poppy enzyme fraction showed the same activity. The rate of reaction was influenced by phenolic compounds and their reaction was controlled by the concentration of H_2O_2 and the presence of ascorbic acid [11]. In this study, we have shown that the oxidation reaction was the major degradation process. Norcodeine and normorphine, the demethylation products of codeine and morphine, were detected in only trace amounts, which prevented any further structural analysis. The dimerization product of morphine, pseudo-morphine, was not found in the isolated capsule system. Ascorbic acid and several other phenolic compounds such as DOPA and *p*-coumaric acid, did not significantly interfere in the conversion of codeine to morphine in our isolated capsule system. The reducing agents in this study were seen to be important factors in controlling the degradation process of morphine and codeine. The use of these reducing agents to regulate the formation of morphine alkaloids should be explored further.

EXPERIMENTAL

Radioactive [^{14}C-methyl]codeine (sp. act. 56 mCi/mmol) was obtained from Amersham. Thebaine, codeine and morphine were gifts from Penick Corporation, NJ. The alkaloids, norcodeine and normorphine were obtained from Applied Science Laboratories. Codeine *N*-oxide and morphine *N*-oxide were synthesized according to previous reports [15]. All other chemicals were analytical grade and from commercial sources. HPLC and alkaloid extraction solvents were from commercial sources and were Mallinckrodt Nanograde or equivalent.

Inoculation of excised capsules. Capsules (1 week after petal fall) were excised 5 cm below the neck of the capsule and sterilized with 7% commercial Clorox for 5 min. After rinsing with sterilized H_2O, capsules were immersed in 10 ml sterilized MS medium (containing 1 mg/l 2,4-D and 0·1 mg/l. kinetin) in vials. Various concns of cofactors and chemicals (such as pyridoxal phosphate, ATP, NAD, *S*-acetyl CoA, $MgCl_2$, H_2O_2) were predissolved in MS medium and filtered through a millipore filter

(0.20 μm). Aliquots of these solns were added to the above incubation to reach the desired concn. The inoculation was placed at constant temp. (25°) and constant light (130 ft-c) overnight.

Extraction and analysis of alkaloids. After inoculation, capsules were rinsed with distilled H_2O and dried by lyophilization over a period of 2 days. Dry capsules were pulverized and then extracted with $CHCl_3$–i-PrOH (3:1) as described before [17]. The dry residue from the extraction was resuspended in 500 μl EtOH. An aliquot (50 μl) of the extract was spotted on a silica TLC plate and developed in Me_2CO–xylene–MeOH–NH_4OH (99:83:10:8). The TLC plate was scanned with a linear analyser, which automatically integrates the radioactivity and records the migration distance of the radioactive labeled alkaloids. The endogenous non-radioactive alkaloids were detected by spraying the TLC plate with Dragendorff reagent [18] after scanning the plate as described above.

Prep. TLC purification of alkaloids. Alkaloids extracted from the capsules were spotted on prep. TLC plates and developed with the solvent system described above. After determining the migration distance, the alkaloids were scraped from the TLC plates and extracted with EtOH. A second solvent system of Me_2CO–H_2O–conc. NH_4OH (80:15:5) was used to purify the alkaloids recovered from the first prep. TLC further. The alkaloids were again recovered from TLC plates by EtOH extraction; these purified alkaloids were analysed by MS. The isolated morphine *N*-oxide and codeine *N*-oxide had the same R_f values (0.35 and 0.45, respectively) as the natural *N*-oxides when they were chromatographed using the same solvent system.

HPLC and MS. HPLC separations were conducted according to a previous report [17]. MS was performed on a quadrupole instrument. A resolution of 1000 was used throughout the study The samples were introduced through the direct evaporation rod. EI spectra were obtained using an electron energy of 70 eV and a source temp. of 150° ± 5°. NH_3 CI spectra were obtained using an electron energy of 150 eV to ionize the reagent gas and a source temp. of 115° ± 5°.

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