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ENZYMIC REDUCTION OF CODEINONE *IN VITRO* CELL-FREE SYSTEMS FROM *PAPAVER SOMNIFERUM* AND *P. BRACTEATUM*

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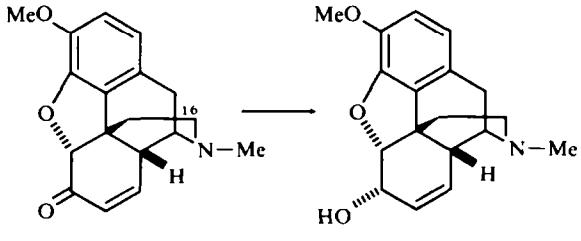
Key Word Index—*Papaver somniferum*; *P. bracteatum*; Papaveracae; poppy; cell-free conversion; codeinone; codeine.

Abstract—Cell-free extracts have been prepared from *Papaver somniferum* which catalyze the reduction of codeinone-[16-^{3}H] to codeine. The methodology for examining this conversion has pointed to conditions for exploring the preparation of suitable enzyme extracts. *P. bracteatum* also yielded a cell-free system which reduced codeinone to codeine, both of which are foreign to this species.

INTRODUCTION

Though identification of the intermediates in the pathway of morphine biosynthesis in *Papaver somniferum* seems nearly complete [1–3], little progress has been made in the study of the enzymic systems which catalyze the individual steps in the biosynthesis of the alkaloid. Even detection of the enzymes responsible has been difficult outside the living plant. Successful *in vitro* conversions of morphinan alkaloids have been claimed in cases where a radioactive precursor has undergone a number of enzymic transformations before the product was isolated [3–6]. Most studies have been performed on the latex of the plant which is tedious to obtain and difficult to handle in large quantities. Only one study has shown the reduction of codeinone (**1**) to codeine (**3**) after a 16 hr incubation in a crude extract from cultured poppy cells [7].

To provide a basis for isolating the enzymes responsible for alkaloid biosynthesis, our studies have utilized cell-free extracts from entire opium plants to demonstrate the specific *in vitro* conversion of codeinone (**1**) to codeine (**2**). Methods of quantitating this conversion at the nmol level were developed to allow the possibility of preparing active enzyme extracts.



1 **2**

Recent studies have indicated that, although codeinone is not accumulated to detectable amounts in *P. bracteatum*, intact plants can reduce injected codeinone to codeine [8,9]. Therefore, an enzyme extract of *P. bracteatum* was made and tested to see if such enzymic activity could be detected.

RESULTS AND DISCUSSION

To achieve high sensitivity and reaction specificity, our assay monitored the conversion of tritiated substrate to product. Codeinone-[16-^{3}H] (**1**) was synthesized and purified free of codeine (**2**) by TLC and HPLC. Its specific activity was determined by liquid scintillation counting and quantitative mass determinations by HPLC.

The tritium labelled codeinone in aqueous solution was mixed with samples of the enzyme extracts from *P. somniferum* containing added NADH. After 15 min incubation, the solutions were acidified and cooled. Unlabelled carrier codeinone and codeine were added and the total alkaloids recovered by extraction. Codeine was purified by TLC and its specific activity determined by liquid scintillation and GLC.

To quantitate the enzymic conversion, the concentrations of endogenous substrate and product needed to be determined, and for this purpose separate samples of the enzyme extract were used. Endogenous codeinone (**1**) was determined by reduction with $NaBT_4$ of known specific activity. Endogenous codeine was determined by an isotope dilution technique. Control experiments for each enzyme extract determined the contamination of codeine with radioactivity from the substrate resulting from non-enzymic processes. These assays were performed on enzyme preparations previously heat-inactivated. Contamination of codeine ranged from 0.5 to 1.5% of the total radioactivity initially present as codeinone-[16-^{3}H].

As a test to demonstrate *in vitro* enzyme activity and assay precision, a bolting plant of *P. somniferum* was homogenized in 150 mM Tris–HCl buffer, pH 7.5. In this enzyme extract the codeine concentration was 48.0 μg/ml, with 0.68 μg/ml of codeinone. The extract was heat-inactivated and treated with NADH and codeinone-[16-^{3}H] (2.41 μg, 8.13 nmol). The recovered codeine contained only 1.47% of the codeinone radioactivity. When an

undenatural enzyme preparation was treated in the same way 26% of the total codeinone was converted to codeine. Smaller volumes of the crude enzyme preparation were assayed and gave reasonably proportional conversions. These results thus clearly demonstrate quantitative and reproducible evidence for the *in vitro* enzymic reduction of codeinone-[16-^{3}H] (**1**) to codeine (**2**) and are presented in Table 1.

The same assay procedure was utilized to develop improved conditions for preparing the reductive enzyme extract. Activity was detectable if the plant was homogenized in either 150 mM Tris–HCl or 150 mM MES [2-(*N*-morpholino)ethanesulfonic acid]/NaOH with pH from 6.3 to 8.0. The optimum pH for the assay was found to be 7.5 over the pH range 6–8.

The possible loss of enzyme activity caused by endogenous polyphenols and phenol oxidases [10, 11] was investigated by the use of Amberlite resins XAD-2 and XAD-4 and insoluble polyvinylpyrrolidone (PVP) to adsorb these compounds: none of these significantly increased the yield of enzyme activity. Attempts to chelate any polyphenols using soluble polyvinylpyrrolidone slightly decreased activity (Table 2). To reduce the possible production of enzyme-inactivating products from the action of phenoloxidases, plant extracts were prepared by homogenization in nitrogen with nitrogen-purged buffers. Despite these precautions, extracts developed discoloration showing that phenoloxidase activity was probably present. Unfortunately, the recommended thiol and sulfite inhibitors of phenoloxidase reacted with codeinone and were thus unsuitable for our assays. Although codeinone was stable in the presence of ascorbic acid, additions of this reagent showed no promoting or stabilizing effect on codeinone reduction. Even on storage at 0° under nitrogen, the crude extracts lost detectable ability to reduce codeinone within 3 hr after homogenization.

Both plant age and plant tissue were examined as variables. A bolting plant was dissected into roots, stems, and leaves, and the tissues assayed separately. All of them showed similar concentrations of codeinone-reducing activity, based on fr. wt of tissue. Thus, crude extracts were later prepared from whole plants.

Plants of various ages were assayed. Codeinone-reducing activity per g tissue increased with plant age: maximum activity was obtained from bolting plants with buds on flowers.

Eight-month-old, non-bolting specimens of *P. bracteatum* were also used to prepare an enzyme extract.

Table 1. The *in vitro* enzymatic reduction of codeinone

Extract vol. (ml)	Codeinone reduced* (nmol)
4, heat inact.†	0.25‡
4	4.42
3	3.73
2	2.36
1	0.50
0.5	0.29

Assay as a function of sample volume from a single extract of *P. somniferum*.

* Amount reduced during entire 15 min assay period.

† Heat-inactivated for control experiment.

‡ Codeine radioactivity was 1.47% of total added as codeinone-[^{3}H].

Table 2. Effects on codeinone-reducing activity by treating extracts with phenol adsorbents

Adsorbent added	Codeinone-[^{3}H] reduced, (%)
None, heat inact	1.1
None	20.2
Amberlite XAD-2	22.8
Amberlite XAD-2	25.6
PVP, insoluble*	17.5
PVP, soluble, 10%†	15.6
PVP, soluble, 1%	16.2

* Polyvinylpyrrolidone; 360 000 MW.

† Grams of polyvinylpyrrolidone (10 000 MW) per ml of extract.

Codeinone-reducing activity per g tissue was definite, although low compared to bolting *P. somniferum*. This result corroborates our previous studies in which radioactivity from labelled codeinone injected into intact *P. bracteatum* plants was recovered in codeine [8]. The summary of these enzymic and control experiments is given in Table 3.

EXPERIMENTAL

Materials. The plant materials, conditions of GLC analyses, and the synthesis of codeinone-[16-^{3}H] were as described previously [8, 12]. All alkaloids were purified on analytical Si gel TLC plates containing UV-fluor. TLC plates for codeinone-[16-^{3}H] purification were developed in $CHCl_3$–MeOH–Et_3N (100:20:1). The recovered codeinone-[16-^{3}H] was further purified by HPLC using a Spectra Physics SP-8000 instrument equipped with a UV detector at 280 nm. Purification was achieved on a 3.2 × 250 mm column packed with 10 μm LiChrosorb RP-18 using an eluting solvent of MeOH–H_2O (10:1) containing 0.01% conc NH_3 at 0.4 ml/min.

Enzyme extracts. All extract prepn was performed in a N_2-filled glove-box at 3°. Buffers were deoxygenated by bubbling N_2 through for several hr. Fresh plants were homogenized in a glass blender with buffer (150 mM Tris–HCl, pH 7.5) using 1.3 ml/g tissue. The homogenate was filtered through Miracloth.

Enzymic conversion of codeinone-[16-^{3}H] to codeine-[16-^{3}H]. In the N_2 box, a 4 ml portion of the enzyme extract was placed in a septum-capped vial. The closed vial was removed from the box and placed in a water bath at 24°. Rapidly, 100 μl of std buffer containing 2 μmol of NADH was injected by syringe. By a second injection, 50 μl 1 mM HCl containing 8.13 nmol codeinone-[16-^{3}H] (1.5 × 10^5 dpm) was added. (The amount of codeinone-[^{3}H] varied from assay to assay but was determined exactly by scintillation analysis of an aliquot of the soln.) The vial was shaken for 15 min and the enzymic reaction was then stopped by adding 50 μl 85% H_3PO_4 and cooling the vial in ice–H_2O. To the soln was added 150 μg each of non-radioactive codeinone and codeine in 100 μl MeOH. The soln was then extracted twice, each with 2 ml $CHCl_3$ containing 0.1% cyclohexanone*. Phase separation was hastened by 1 min spins in a clinical centrifuge. The organic phases were discarded and the remaining aq. fraction was cooled in ice–H_2O. Basification with 300 μl conc NH_4OH was followed by 3

* To reduce the possibility of Oppenauer equilibration of codeinone-[^{3}H] to codeine-[^{3}H], ketonic solvents and TLC plates at room temp. were used in codeine purification.

Table 3. Reduction of codeinone to codeine by various extracts of *P. somniferum* and *P. bracteatum*

Tissue	Age of plant (days)	Extract A or HI*	Codeinone-[^{3}H] reduced (%)	Codeinone (nmol per g tissue)
P. somniferum				
Stems	125	HI	1.4	
	125	A	18.0	6.6
Leaves	125	HI	1.0	
	125	A	25.0	5.2
Roots	125	HI	1.2	
	125	A	12.0	8.5
Whole plant	27†	HI	1.4	
	27	A	14.0	5.8
	65‡	HI	1.6	
	65	A	18.0	9.6
	130§	HI	1.1	
	130	A	20.1	13.3
P. bracteatum				
Whole plant	265¶	HI	1.0	
	265	A	5.2	0.9

* Active (A) or heat-inactivated (HI) extracts.
† Non-bolting.
‡ Beginning to bolt, no bud.
§ Flowering.
¶ Non-bolting.

extractions as above. The organic phases were removed, combined, and evapd under N_2. Traces of H_2O were removed under vacuum. The residue was resuspended in 75–100 μl Me_2CO and spotted on a TLC plate at room temp. which was developed 3 × in Me_2CO containing 0.5% Et_3N. The codeine band was scraped from the plate and the codeine-[16-^{3}H] recovered by elution with CH_2Cl_2–MeOH–cyclohexanone (40:10:1). To the eluted codeine-[16-^{3}H] was added 150 μg of non-radioactive codeinone in the eluting solvent, and the solution was evapd under N_2, dissolved in Me_2CO, and spotted on another TLC plate. This plate was developed once in Et_3N–$CHCl_3$–MeOH (10:1:1). The codeine band was scraped off and the codeine-[16-^{3}H] freed from the silica by elution with CH_2Cl_2–MeOH (4:1). The soln was evapd under N_2 and dissolved in 500 μl MeOH. Codeine concentraction was determined by GLC and the rest of the sample was used for liquid scintillation analysis.

To assay less than 4 ml of extract, the desired vol. of extract was diluted with std. buffer to give a total vol. of 4 ml. Control assays were performed on extract samples inactivated by heating the sample in boiling H_2O for 15 min. To calculate the total codeinone reduced, T (μg):

$$T = \left(\frac{u + \mathrm{EC}}{q}\right)\left(\frac{r}{v}\right)(z + 4\,\mathrm{ECO}),$$

where u = carrier codeine (μg); EC = endogenous codeine (μg—see below); q = recovered codeine (μg); r = dpm of recovered codeine; v = dpm added as codeinone-[^{3}H]; z = amount of codeinone-[^{3}H] (μg); ECO = endogenous codeinone (μg—see below).

Endogenous codeine. To 4 ml of enzyme extract was added 16.8 μg (6.30 × 10^4 dmp) of carrier codeine-[16-^{3}H] in 2 ml MeOH. Codeine was recovered and analyzed for sp. act. by the methods described above. Using the radioactivity recovery to determine a normalization factor, the endogenous codeine, EC (μg) in the extract before alkaloid purification could be calculated. Thus:

$$\mathrm{EC} = \left(\frac{bc}{a}\right) - d,$$

where a = recovered dpm; b = total added dpm; c = recovered codeine (μg); d = carrier codeine (μg).

Endogenous codeinone. $NaBT_4$, previously titrated as described below, was suspended in 1 ml MeOH. To this was added 1.00 ml enzyme extract and the mixture shaken for 10 min. The excess $NaBT_4$ was destroyed by adding 1 ml of Me_2CO and the mixture was concd under a stream of N_2. To the remaining soln were added 2 ml std buffer then 150 μg non-radioactive codeine in 100 μl MeOH, and the codeine was then isolated and purified as above. The total production of codeine-[16-^{3}H] was calculated from the sp. act. of the purified codeine and the known masses of endogenous and added carried codeine. Then the sp. act. of the $NaBT_4$ allowed determination of the concn of endogenous codeinone, ECO (μg).
Thus:

$$\mathrm{ECO} = \left(\frac{e}{s \cdot f}\right)\left(g + \frac{\mathrm{EC}}{4}\right),$$

where e = recovered dpm; s = specific activity of $NaBT_4$ reduction (see below); f = recovered codeine (μg); g = added carrier codeine.

Specific activity of $NaBT_4$. $NaBT_4$ (*ca* 1.5 Ci/mmol) was dissolved in 0.2% NaOH. Equal portions of the soln, each containing *ca* 1 μmol of $NaBT_4$, were placed in vials convenient for the determination of endogenous codeinone. The water was removed by evapn *in vacuo* to leave stable $NaBT_4$ residues in the vials. To determine the sp. act. by codeinone reduction, S, (as dpm incorporated into 1 μg codeinone by reduction to codeine) the

endogenous codeinone determination was performed on two fresh plant extracts; one neat and one to which was added 1.24 μg of codeinone/ml plant extract. The difference between the radioactivity yields of total codeine-[16-^{3}H] from these two determinations represented the incorporation of tritium by reduction of the added codeinone. The results agreed with the determination performed on 1.24 μg codeinone in buffer only. Thus:

$$S = \frac{\left[\left(\frac{hm}{n}\right)_x - \left(\frac{hm}{n}\right)_y\right]}{p},$$

where h = recovered dpm; m = carrier plus endogenous codeine; n = recovered codeine, x = from extract with exogenous codeinone; y = from neat extract; p = amount of exogenous codeinone (μg).

Effects of pH on extract activity. A bolting plant was ground in 150 mM MES, pH 7.0. From the filtered extract, 10 ml portions were adjusted to desired pHs with NaOH or HCl solns. The adjustments were performed in the N_2 box at 3° with a calibrated pH meter. The portions were then assayed. Similar tests were performed with 150 mM Tris as the grinding buffer.

Attempts to remove or deactivate polyphenols. Amberlite XAD-2 and XAD-4 were washed several times with H_2O, equilibrated with buffer, and drained. Insoluble (360 000 MW) PVP was boiled in 1 N HCl for ½ hr, filtered, washed with H_2O, neutralized with NaOH, washed with H_2O then buffer, and drained. Buffer solutions of 10 000 MW PVP (500 mg/ml and 50 mg/ml) were prepared. The reagents were placed in the N_2 box. Enzyme extract aliquots (8 ml) were combined with 1 g (wet wt) of adsorbent or 2 ml of reagent soln, or, for the control experiment, 2 ml of std buffer. The resulting mixtures were shaken for 3 min and filtered through glass wool to give treated enzyme extracts which were assayed in normal fashion.

Stabilization of enzyme activity with ascorbic acid. Into separate flasks were placed 60 mg of sodium ascorbate, dissolved in 2 ml of buffer, and 600 mg of sodium ascorbate in 2 ml buffer. The control flask contained 2 ml of buffer only. In the N_2 box, 28 ml portions of enzyme extract were added to each flask to give solns of 0, 10 and 100 mM ascorbic acid. The solns were kept in the cold N_2 box and aliquots assayed at 15, 40, 75, 135 and 195 min.

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