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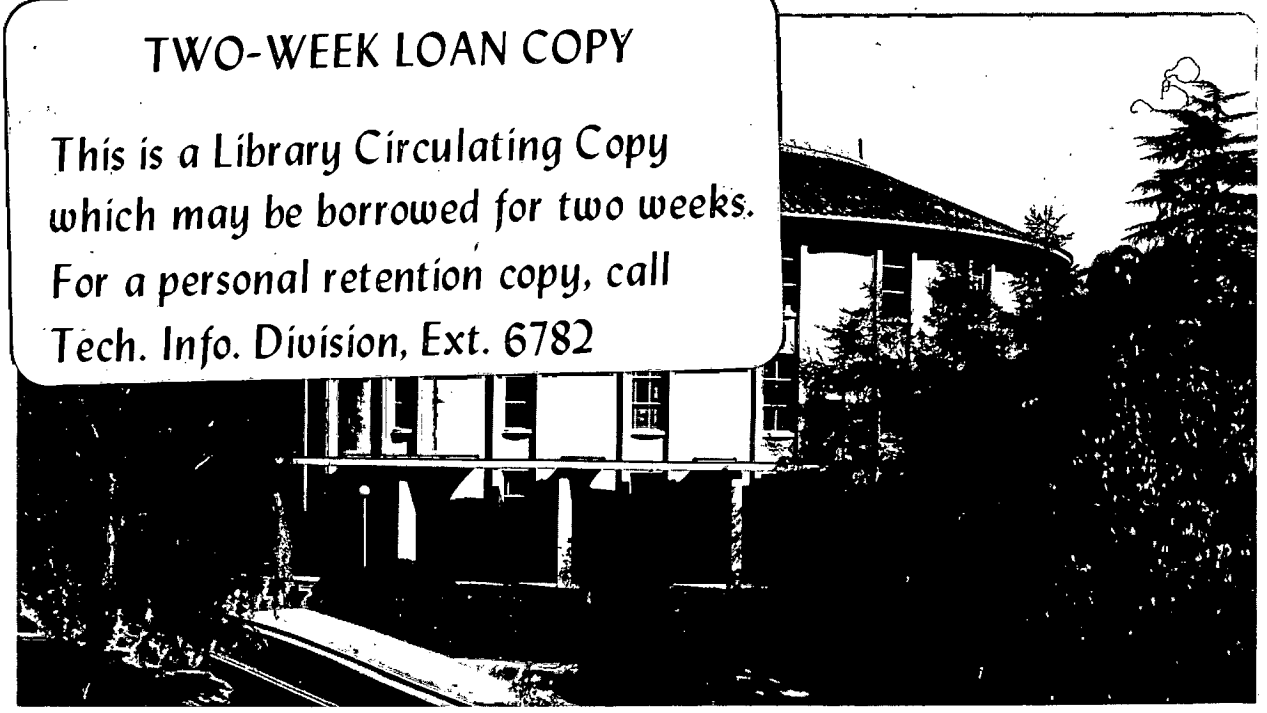

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ENZYMIC REDUCTION OF CODEINONE IN VITRO: CELL-FREE SYSTEMS FROM PAPAVER SOMNIFERUM AND P. BRACTEATUM

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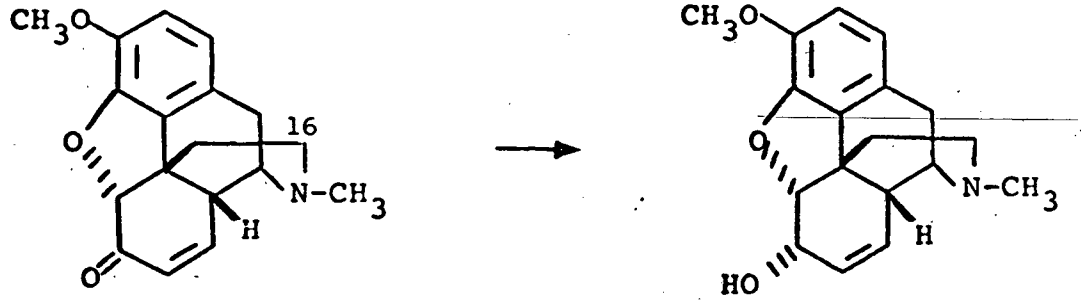
Key Word Index - Papaver somniferum; Papaver bracteatum; Papaveracae; poppy; cell-free conversion; codeinone; codeine.

Abstract - Cell-free extracts have been prepared from Papaver somniferum which perform the reduction of [16-^{3}H]codeinone to codeine. Methodology for quantitating this conversion has allowed conditions for preparing the enzyme extracts to be explored. From P. bracteatum, a cell-free system was also prepared which reduced codeinone to codeine, both of which are foreign alkaloids to this species.

INTRODUCTION

Through 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 perform the alkaloid transformations. In other than living plants, even detection of these enzymes has been difficult. Successful in vitro conversions of the morphinan alkaloids have been claimed in cases where a radioactive precursor has undergone a number of enzymic transformations before the product has been isolated [3-6]. Most studies have been performed on plant latex, which is tedious to obtain and difficult to handle in large quantities. A single study has detected the reduction of codeinone after a 16 hour incubation in a crude extract from cultured poppy cells [7].

To provide a more direct basis for the ultimate goal of 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 nmole conversions were developed to allow the possibility of optimizing the preparation of active enzyme extracts.



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

RESULTS AND DISCUSSION

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

[16-^{3}H]Codeinone in aqueous solution was mixed with samples of the enzyme extracts containing added NADH. After 15 min incubation periods, the enzymic conversions were halted by acidification and cooling. Unlabelled carrier codeinone and codeine were added and the alkaloids recovered by organic solvent 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 also had to be determined, and for this purpose separate samples of the enzyme extract were used. Endogenous codeinone was determined by reduction with $NaBT_4$ of known specific activity for this reduction. 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 [16-^{3}H]-codeinone.

As a test to demonstrate in vitro enzyme activity and assay precision, an 83 g bolting plant was homogenized in 150 mM Tris·HCl buffer, pH 7.5, to yield 115 ml of enzyme extract. Using 3 ml of this extract, the endogenous codeine concentration was determined to be 48.0 µg/ml, whereas a 1 ml sample of the extract was found to contain 0.68 µg (2.15 nmoles) of endogenous codeinone. A four ml sample of the extract was heat-inactivated for the control experiment. This sample was treated with NADH and [16-^{3}H]codeinone (2.41 µg, 8.13 nmoles). Recovered codeine from the assay was contaminated with only 1.47% of the [16-^{3}H]-codeinone radioactivity. When 4 ml of the live enzyme preparation was treated with the same quantities of NADH and [16-^{3}H]codeinone, 26.1% (4.42 nmoles) of the total codeinone (4x2.15 + 8.13; 16.73 nmoles) was converted to codeine. Smaller volumes of the 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 [16-^{3}H]codeinone and are presented in Table 1.

Table 1 here

The same assay procedure was utilized to develop improved conditions for preparing the 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

ranging from 6.3 to 8.0. Optimum pH for the assay was found to be 7.5 over the pH range 6 to 8 in 0.5 unit increments. This may represent the combined effects of pH on both enzyme turnover rate and enzyme stability in these crude extracts.

fn 10,11 Suggestions in the literature [10,11] were followed in addressing problems of the loss of enzyme activity caused by endogenous polyphenols and phenol oxidases. Attempts to remove polyphenols by absorption on Amberlite resins XAD-2 and XAD-4 and insoluble polyvinylpyrrolidone (PVP) did not significantly increase the yield of enzyme activity. Attempts to chelate polyphenols with soluble polyvinylpyrrolidone slightly decreased activity. Table 2 here These results are summarized in Table 2.

To reduce the production of enzyme inactivating products from phenol oxidases, the plant extracts were prepared by homogenization in a nitrogen atmosphere with nitrogen-purged buffers. Despite these precautions, the extracts developed discoloration as evidence of phenol oxidase activity. Recommended thiol and sulfite inhibitors reacted with codeinone and were thus unsuitable. Although codeinone was stable in the presence of ascorbic acid, additions of this reagent showed no promoting or stabilizing effect on codeinone reduction activity. Even with storage at 0° under nitrogen, these crude extracts lost detectable ability to reduce codeinone within 3 hr after plant homogenization.

Both plant age and plant tissue were examined as variables. A bolting plant was simply dissected into roots, stems, and leaves, and these tissues were assayed separately. All tissues showed similar concentrations of codeinone reducing activity, based on

fresh weight of tissue. Thus, our crude extracts were later prepared from whole plants.

Plants of various ages were assayed. Codeinone-reducing activity per gram 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 used to prepare an enzyme extract. Codeinone-reducing activity per gram 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.

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EXPERIMENTAL

Materials. The plant materials, conditions of GLC analyses, and the synthesis of [16-^{3}H]codeinone have been described previously [8,12]. All alkaloids were purified on analytical silica gel TLC plates containing UV fluor. TLC plates for [16-^{3}H]codeinone purification were developed in $CHCl_3/CH_3OH/Et_3N$ (100/20/1). The recovered [16-^{3}H]codeinone was further purified by HPLC using a Spectra Physics SP-8000 instrument equipped with a UV detector set at 280 nm. Purification was achieved on a 3.2x250 mm column packed with 10 μ LiChrosorb RP-18 using an eluting solvent of CH_3OH/H_2O (10/1) containing 0.01% conc NH_3 flowing at 0.4 ml/min.

Enzyme extracts. All extract preparation was performed in a nitrogen-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 std buffer (150 mM Tris·HCl, pH 7.5) using 1.3 ml buffer per 1 g tissue. The homogenate was filtered through Miracloth to give the enzyme extract.

Enzymic conversion of [16-^{3}H]codeinone to [16-^{3}H]codeine. In the nitrogen 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 μmoles of NADH was injected by syringe. By a second injection, 50 μl of 1 mM HCl containing 8.13 nmoles of [16-^{3}H]codeinone (1.56×10^5 dpm) was added. (The amount of ^{3}H-codeinone varied from assay to assay but was determined exactly by scintillation analysis of an aliquot of the alkaloid soln.). The vial was shaken for 15 min and the enzymic reaction was then

stopped by adding 50 µl of 85% H_3PO_4 and cooling the vial in ice-water. To the soln was added 150 µg each of non-radioactive codeinone and codeine in 100 µl CH_3OH. The soln was then extracted fn 13 2 times, each with 2 ml of $CHCl_3$ containing 0.1% cyclohexanone [13]. Phase separation was hastened by 1 min spins in a clinical centrifuge. The organic phases were discarded and the remaining aqueous fraction was cooled in ice-water. Basification with 300 µl conc NH_4OH was followed by 3 extractions as above. The organic phases were removed, combined, and evaporated under N_2. Traces of water were removed under vacuum. The residue was resuspended in 75-100 µl of acetone and spotted on a TLC plate at room temp which was developed 3 times in acetone containing 0.5% Et_3N. The codeine band was scraped from the plate and the [16-^{3}H]codeine recovered by elution with CH_2Cl_2/CH_3OH/cyclohexanone (4/1/0.1%). To the eluted [16-^{3}H]codeine was added 150 µg of non-radioactive codeinone in the eluting solvent, and the solution was evaporated under N_2, dissolved in acetone, and spotted on another TLC plate. This plate was developed once in $Et_3N/CHCl_3/CH_3OH$ (10/1/1). The codeine band was scraped off and the [16-^{3}H]codeine freed from the silica by elution with CH_2Cl_2/CH_3OH (4/1). The soln was evaporated under N_2 and dissolved in exactly 500 µl CH_3OH. Codeine concentration 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 volume of extract was diluted with std buffer to give a total volume of 4 ml. Control assays were performed on extract samples inactivated by heating the sample in boiling water for 15 min.

To calculate the total codeinone reduced, T (μg):

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

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

Endogenous codeine. To 4 ml of enzyme extract was added 16.8 μg (6.30×10^4 dpm) of carrier [16-^{3}H]codeine in 2 ml CH_3OH. Codeine was recovered and analyzed for specific activity 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:

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

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 CH_3OH. 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 acetone and the mixture was concentrated under a stream of N_2. To the remaining solution were added 2 ml std buffer then 150 μg non-radioactive codeine in 100 μl CH_3OH, and the codeine was then isolated and purified as above.

The total production of [16-^{3}H]codeine was calculated from

the specific activity of the purified codeine and the known masses of endogenous and added carried codeine. Then the specific activity of the $NaBT_4$ allowed determination of the concentration of endogenous codeinone, ECO (μg).

Thus:

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

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

Specific activity of $NaBT_4$. $NaBT_4$ (approx. 1.5 Ci/mmole) was dissolved in an aqueous solution of 0.2% NaOH. Equal portions of the soln, each containing about 1 μmole of $NaBT_4$, were placed in vials convenient for the determination of endogenous codeinone. The water was removed by evaporation in vacuo to leave stable $NaBT_4$ residues in the vials.

To determine the specific activity 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 [16-^{3}H]codeine 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 distilled water, equilibrated with buffer, and drained. Insoluble (360,000 MW) PVP was boiled in 1N HCl for 1/2 hr, filtered, washed with distilled water, neutralized with NaOH, washed with distilled water 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 nitrogen 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 nitrogen box, 28 ml portions of enzyme extract were added to each flask to give solns of 0, 10 mM, and 100 mM ascorbic acid. The solns were kept in the cold nitrogen box and aliquots assayed at 15, 40, 75, 135 and 195 min.

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

Table 1. *In vitro* enzymatic reduction of codeinone. Assay as a function of sample volume from a single extract of *P. somniferum*.

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

* Amount reduced during entire 15 min assay period.

† Heat inactivated for control experiment.

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

Table 2. Effects on enzyme activity by treating extract aliquots with phenol adsorbents.

Adsorbent added	^{3}H-Codeinone 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.

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

Species	Tissue	Age of plant, days	Extract A or HI*	^{3}H-Codeinone reduced, %	Codeinone, nmoles per g tissue
P. somniferum					
	stems	125	HI	1.4	
	stems	125	A	18.0	6.6
	leaves	125	HI	1.0	
	leaves	125	A	25.0	5.2
	roots	125	HI	1.2	
	roots	125	A	12.0	8.5
	whole plant	27†	HI	1.4	
	whole plant	27	A	14.0	5.8
	whole plant	65‡	HI	1.6	
	whole plant	65	A	18.0	9.6
	whole plant	130§	HI	1.1	
	whole plant	130	A	20.1	13.3
P. bracteatum					
	whole plant	265¶	HI	1.0	
	whole plant	265	A	5.2	0.9

* Active (A) or heat inactivated (HI) extracts.

† Non-bolting.

‡ Beginning to bolt, no bud.

§ Flowering

¶ Non-bolting.

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