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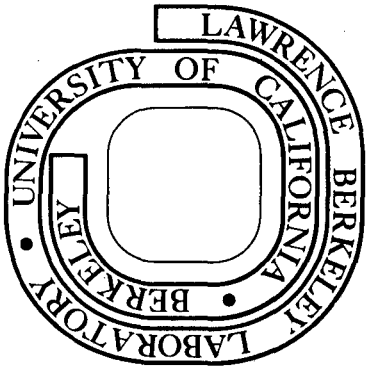

DISCLAIMER

MORPHINAN ALKALOIDS IN PAPAVER BRACTEATUM. BIOSYNTHESIS AND FATE.

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Key Word Index - Papaver bracteatum; Papaveracae; biosynthesis; codeinone; codeine; morphine; reticuline; dehydroreticulinium; chloride; thebaine; oripavine; northebaine.

Abstract - The known metabolic pathway for hydrophenanthrene alkaloids in P. somniferum has been examined for occurrence in P. bracteatum, a species reported to contain thebaine but no codeine or morphine. 1,2-Dehydroreticulinium-[3-^{14}C] chloride and (±)-reticuline-[3-^{14}C] were fed to P. bracteatum plants and both were incorporated, the former into reticuline and thebaine and the latter into thebaine, suggesting that thebaine biosynthesis is the same in the two species. Studies of the natural abundance of morphinan alkaloids in P. bracteatum and the results from feeding codeinone-[16-^{3}H] and codeine-[16-^{3}H] indicate that this species can reduce codeinone to codeine but can not perform either of the demethylations to produce codeinone or morphine. Fed thebaine-[16-^{3}H] was substantially metabolized but not by pathways that involved demethylations to either oripavine or northebaine.

INTRODUCTION

The frequency of occurrence of thebaine-containing members of the genus Papaver [1-5] invites the comparison of metabolic pathways for hydrophenanthrene alkaloids among the different species. Most attention has focused on P. somniferum [6-8] in which tyrosine is the precursor for the metabolic sequence of compounds 1 through 9. However, in most species containing thebaine (5), no codeine (7) or morphine (8) have been detected. For these species, the known scheme for thebaine metabolism may not be applicable.

Investigations presented here were performed on P. bracteatum, in which reportedly thebaine is the major alkaloid but codeine and morphine are absent [5]. The relative abundances to thebaine in the plant of codeine, morphine, and the other demethylation derivatives of thebaine, oripavine (10) and northebaine (11), were determined by analysis of solvent extracts by gas liquid chromatography (GLC).

Injections into the plant of labeled alkaloids were followed by searches for conversions to the subsequent compounds suggested by the P. somniferum pathway. 1,2-Dehydroreticulinium-[3-^{14}C] chloride (1) and reticuline-[3-^{14}C] (2) were fed for investigation of thebaine biosynthesis. Codeinone-[16-^{3}H] (6) and codeine-[16-^{3}H] (7) were fed for examination of the thebaine pathway to morphine. Similar experiments investigated the possibility that thebaine metabolism involved direct demethylation to oripavine or to northebaine.

RESULTS

Natural abundance of thebaine and demethylated derivatives in P. bracteatum

Standard procedures for alkaloid extraction on 1.44 kg (fresh weight) from 48 plants, 5 to 7 months old, provided phenolic and non-phenolic alkaloid fractions which were analyzed by GLC. Calibration with pure alkaloid standards established the presence of 820 mg of thebaine (0.06% of the fresh weight) and 4 mg of oripavine (0.5% w/w of thebaine). No codeine, morphine, or northebaine were detected. Experimentally determined limits of detection of these alkaloids were 1 ppm, 1 ppm, and 50 ppm, respectively per unit of thebaine.

Thebaine biosynthesis

(±)-Reticuline-[3-^{14}C] (2) and 1,2-dehydroreticulinium-[3-^{14}C] chloride (1) were synthesized and injected into 5 month old plants. The plants were allowed to grow for specific periods of time and the labeled alkaloids extracted from them using carried dilution techniques.

Both reticuline and 1,2-dehydroreticulinium chloride were found to be incorporated into thebaine (5) (Table 1). 1,2-Dehydroreticulinium chloride was also found to be incorporated into reticuline. Previously, Neubauer [9] demonstrated that tyrosine-[2-^{14}C] is incorporated by P. bracteatum into thebaine in 1.3 to 2.5% yield. This evidence together with our data confirms that the biosynthetic pathway to thebaine in P. somniferum is also common to P. bracteatum.

The incorporation rates of reticuline and 1,2-dehydroreticulinium chloride in P. bracteatum are substantially less than those found for P. somniferum [10]. Though the pathways involve the same chemical intermediates, the characteristics of the enzymes involved may be noticeably different. On the other hand, corresponding enzymes in the two species may be essentially the same but in lower concentrations in P. bracteatum.

Thebaine metabolism to morphine

Preliminary experiments showed that the levels of incorporation to be anticipated in some cases might be very low. Thus, ^{3}H-labelled morphinan compounds of high specific activity were produced [10]. Since even small but definite biosynthetic conversions of the alkaloids had potential interest, methods were developed for the preparation of highly purified materials. Purification procedures were tested with inactive compounds and radioactive contaminants. These methods and results are presented in Table 2.

Codeinone-[16-^{3}H] and codeine-[16-^{3}H] were fed to P. bracteatum plants (Table 3, experiments 1 and 2). The lengths of growth periods after injection and before harvest had been determined from preliminary studies of metabolism rates with unlabeled alkaloids. These preliminary studies of alkaloid metabolism rates also suggested that morphine is metabolized in P. bracteatum slightly

more rapidly than is codeine. This implied the complication that morphine produced in the plant from injected codeine may be mostly lost by further metabolism. In view of this, an additional experiment (experiment 3, Table 3) was performed in which unlabeled morphine was injected at intervals into the codeine-fed plants to create andmaintain a morphine pool. Metabolically produced morphine would thus be diluted and suffer less loss than in the experiment in which codeine alone was injected.

The results show that P. bracteatum can perform only one of the three established transformations of the post-thebaine pathway found in P. somniferum, that of the reduction of codeinone to codeine. For this reaction, the rate is comparable to that found in P. somniferum. This ketone reduction step is basically similar to the reduction of salutaridine (3) to salutaridinol (4), which is observed in thebaine biosynthesis in P. somniferum [8] and, from the previous conclusions of this paper, is implied to also occur in P. bracteatum. Perhaps the reductase responsible for salutaridine reduction is not totally specific and thus allows P. bracteatum to deal with the foreign substance codeinone.

In P. bracteatum, the feedings of labeled codeine showed that demethylation to morphine was insignificant. Though 50 and 65% of the injected codeine in the experiments of concern were metabolized, little activity was found in the morphine. The close agreement in the two labeled morphine recovery figures suggests that the observations were not merely the consequence of further morphine metabolism. These results and the absence of naturally-occurring morphine in crude extracts indicate morphine is not a short-lived

intermediate but is a foreign compound to P. bracteatum.

The pathway from thebaine to codeinone also suffers blockage. In view of the facts that codeinone is rapidly converted to codeine, that codeine is metabolized at only a modest rate, and that the isolation techniques give close to quantitative recovery of codeine, any significant biosynthesis of codeinone from thebaine would have been indicated by the discovery of codeine in the previous studies on the natural codeine content of P. bracteatum. Thus, this step, thebaine to codeinone, is clearly absent.

Thebaine metabolism to oripavine and northebaine

Since, in P. somniferum, thebaine undergoes sequential demethylations at the O-6, O-3, and N-17 positions, we considered the possibility that thebaine metabolism in P. bracteatum may also be characterized by demethylation, but occurring in a different sequence. Initial demethylation at the O-3 position would give oripavine (10), while initial N-demethylation would lead to northebaine (11). Even though northebaine was not detected as a natural product in P. bracteatum and oripavine was found only in low concentration compared to thebaine, there was the possibility of rapid metabolism and turnover through these compounds.

Oripavine and oripavine-[16-^{3}H] were synthesized from morphine and morphine-[16-^{3}H] as described [11]. Northebaine and northebaine-[16-^{3}H] were obtained by the demethylation of thebaine [12]. Quantitative studies to develop methods of separation and purification of oripavine and northebaine from labeled thebaine are presented in Table 2.

In each of several experiments, thebaine-[16-^{3}H] was injected into 5 to 7 month old plants. After 5 days, the plants were harvested and the alkaloids isolated to determine what quantity of labeled thebaine had been metabolized and what proportion of the metabolized thebaine had been incorporated into oripavine or northebaine. Thebaine-[16-^{14}C] and known quantities of cold oripavine and northebaine were used as carriers to accurately determine losses through isolations and purifications.

Significant metabolism of labeled thebaine was observed in each of the experiments. Rates ranged from 2 to 3 mg per plant over 5 day periods. However, little of the metabolized thebaine was recovered as oripavine or northebaine (Table 3, experiments 4 and 5). To check the possibility of rapid secondary metabolism in these experiments, labeled oripavine and labeled northebaine were fed and the quantities metabolized after 5 days determined. Two quantities covering a 100 fold mass range for each alkaloid were tested to check the possibility of mass-dependent inhibition of metabolic enzymes. The results (Table 3, experiments 6-9) suggest that oripavine and northebaine metabolisms are sufficiently slow to have allowed detection of significant thebaine-[16-^{3}H] incorporations in the prior feeding experiments, had such incorporations occurred.

The possibility exists, however, that the sites of alkaloid metabolism are compartmented and the metabolism rates from these experiments really reflect transportation rates. If such compartmentation exists, it seems that it would have to occur within the latex itself. Studies with P. somniferum have suggested active

alkaloid metabolism in the latex, perhaps in organelles [13]. In addition, since both oripavine and northebaine are slowly but definitely metabolized, transportation of these alkaloids is not specifically inhibited. One may speculate that resistance to transportation would then be due to availability of specific transportation sites. Contrary to our observations, this hypothesis would suggest that significantly greater proportions of the small quantities of injected alkaloids should have been metabolized as compared to the metabolized proportions in the experiment involving large alkaloid quantities. Thus we are left with the conclusion that, although thebaine is metabolized at a reasonable rate in P. bracteatum, neither O-6 nor O-3, nor N-demethylation is a major initial step of this metabolism.

The metabolism of thebaine and oripavine, naturally occurring in P. bracteatum, as well as the metabolism of codeine, morphine, and northebaine, either foreign or trace components, suggests that there may exist a non-specific enzyme system which can metabolize a variety of thebaine-like compounds. Since the metabolism rates for all of these alkaloids are not strikingly different, the enzyme may have similar affinities for these compounds. The observation that roughly half of the injected oripavine and northebaine is metabolized in 5 days whether 5 or 0.05 mg per plant is fed supports the proposal that an injected alkaloid disperses throughout a larger general alkaloid pool and is processed randomly by an enzyme with little specificity among the morphinan alkaloids.

EXPERIMENTAL

Plant materials. P. bracteatum were grown from seeds obtained from Dr. D. Lavie, Weizman Institute, to whom we are grateful. P. somniferum L. var. alba were started from seeds of USDA No. 40. All plants were sprouted and grown in a greenhouse with supplementary lighting to give a daily light period of 15 hrs.

Preparation of labeled alkaloids. 1,2-Dehydroreticulinium chloride (1) and (±)-reticuline (2) labeled at the 3 position with either ^{14}C or ^{3}H were synthesized in these laboratories [10]. Feeding 1,2-dehydroreticulinium-[3-^{3}H] to P. somniferum gave after 3 days metabolism thebaine-[16-^{3}H], codeine-[16-^{3}H], and morphine-[16-^{3}H] in 3.4, 1.8, and 2.2% yields respectively. These were isolated by the extraction procedures and further purified by sequential pTLC separations detailed below.

Codeinone was prepared from codeine by Seki's modification of the Oppenauer oxidation [14]. Oripavine was synthesized from morphine by the method of Barber and Rapoport [11], and thebaine was demethylated by known procedures [12] to give northebaine.

Feeding of alkaloids. The portion of alkaloid to be fed was dissolved in 0.4 ml 1M H_3PO_4, the pH adjusted to 5.5-6.5 with sat. K_2CO_3, and the solution diluted with water to give 0.7 ml total volume. The solution was loaded for injection into gas-tight syringes because the high turgor pressures of these plants caused standard syringes to leak. The plants were readied simply by washing the roots free of soil. Injections were then made into the hypocotyls and steady deliveries of the feeding solution were made over 1.5 hr periods using motorized syringe pumps. Afterwards,

the plants were allowed to grow in aerated nutrient solutions until harvest. Freshly harvested plants were either extracted directly or frozen and stored at -20°C until used.

To assess how much of the labelled alkaloid was actually delivered into the plants, residual solution left in the vials and syringes was collected and analyzed. Leakage of the fed solution from the wound after injection was collected either by covering the wound with a paper bandage until harvest, or, more conveniently, by keeping the wound submerged in the root nutrient solution. Activity analysis of the paper bandages by combustion procedures or direct liquid scintillation of the root solution showed that the wounds often leaked up to 10% of the injected solution.

Extraction procedures. As much as 200 g (fresh weight) of plant material was frozen in liquid N_2 and blended in a steel container to give finely chopped material. Carrier alkaloids were added, followed by 30 ml 10% K_2CO_3 and 400 ml of n-butanol/benzene, 1/1. Blending, decanting of the organic solution, and repeating the butanol/benzene addition to effect four extractions completely separated the alkaloids. The organic solution was extracted with 4 x 100 ml 1M H_3PO_4, the aqueous fraction was basified to pH 13 using 8M KOH with continuous cooling to keep the solution below room temperature at all times, and this solution was extracted with 5 x 100 ml $CHCl_3$ to give the non-phenolic fraction. Adjustment of the pH to 8.6 with 1M H_3PO_4 and cooling followed by extraction with 5 x 100 ml $CHCl_3$/isopropanol (IPA), 3/1, gave the phenolic alkaloid fraction. The organic phases were

dried over anhydrous Na_2SO_4, filtered, and evaporated.

Alkaloid purifications. A specific combination of steps were used to purify a particular alkaloid. The steps, as designated by letter symbols in Table 2 were: (a) standard plant extraction; (b) chromatography on a 60 x 2 cm column of 106 g basic Woelm Alumina (III) in benzene using successively as eluent 250 ml of benzene/$CHCl_3$, 9/1, 400 ml of benzene/$CHCl_3$/IPA, 88.5/10/1.5, and 600 ml of benzene/$CHCl_3$/IPA/CH_3OH, 87.5/10/1.5/1: (c) the impure material was dissolved in benzene and shaken for 1.5 hrs with 1M $NaHSO_3$ at 25°C under N_2. The aqueous layer was separated, basified with sat. K_2CO_3 to pH 8.5, and extracted with benzene. The next steps involved prep TLC on plates of Camag silica with UV phosphors or on Brinkmann SIL G-UV_{254} plates. TLC steps, by solvent systems, were: (d) toluene/CH_3OH/Et_3N, 4/1/1%; (e) toluene/CH_3OH/Et_3N, 3/1/1%; (f) 4 x development in acetone containing 0.5% conc. ammonia.

In the thebaine biosynthesis experiments, reticuline was purified by the sequence of steps a, e, d and thebaine by a, d, d. Each product was one component by TLC and greater than 99.5% pure by GLC.

Alkaloidal starting materials for synthesis were purified with the following steps: thebaine, a, b, d; codeine, a, b, e; morphine, a, e, e. Purifications of alkaloids isolated after metabolism in the thebaine-metabolism experiments are summarized in Table 2.

Analyses. GLC analyses were performed with 2 m x 3 mm glass columns packed with 3% OV-17 on Varaport 30 (Varian Aerograph),

100-200 mesh. Column preparation included purging overnight at 300°C with He, and then treatment at 250°C with 400 mg morphine in $CHCl_3$, introduced by several injections. The determinations were made at 230°C or 250°C on an instrument equipped with FID.

Radioactivity in the metabolic products was determined by adding known amounts of carrier alkaloids to the purverized plant material before extraction, performing the isolations and purifications, and counting by liquid scintillation techniques representative portions of the purified alkaloids. In the case of the thebaine-metabolism experiments, ^{14}C-labelled thebaine was used as carrier material to avoid complication of the analyses with the large quantities of cold thebaine from the plant material. The ^{14}C and ^{3}H in these samples were separated and prepared for counting by combustion in a Packard Sample Oxidizer, Model 306.

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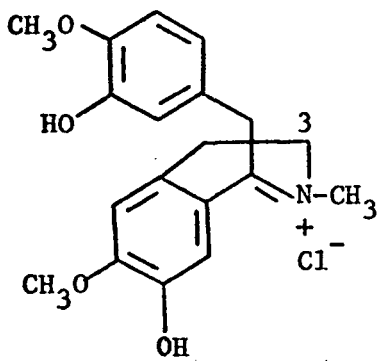

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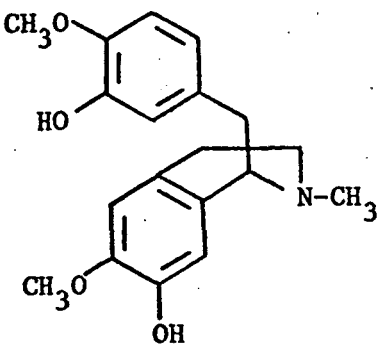

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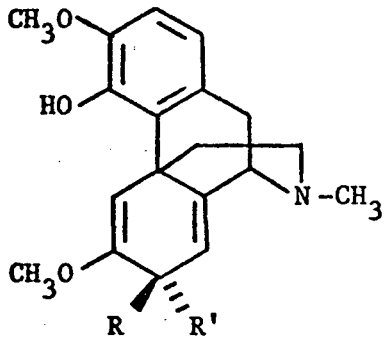
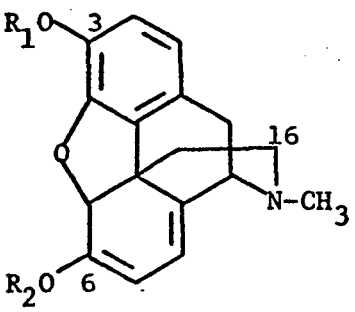


3, R,R'=O

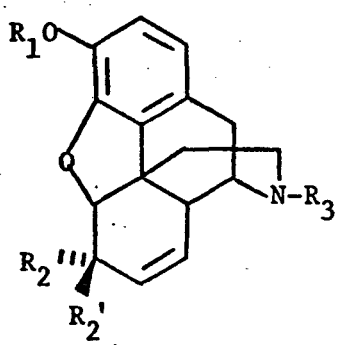
4, R=OH, R'=H



5, $R_1=R_2=R_3=CH_3$

10, $R_1=H$, $R_2=R_3=CH_3$

11, $R_1=R_2=CH_3$, $R_3=H$



6, $R_1=R_3=CH_3$, $R_2,R_2'=O$

7, $R_1=R_3=CH_3$, $R_2=OH$, $R_2'=H$

8, $R_1=R_2'=H$, $R_2=OH$, $R_3=CH_3$

9, $R_1=R_2'=R_3=H$, $R_2=OH$

Table 1. Thebaine biosynthesis. Incorporation of (±)-reticuline-[3-^{14}C] (2) and 1,2-dehydroreticulinium-[3-^{14}C] chloride (1) by P. bracteatum*

Precursor	Metabolism Time	% Precursor Recovered	% Incorporation Reticuline	Thebaine
1,2-Dehydroreticulinium-[3-^{14}C] chloride	20 hr	†	0.5	0.8
	3 days		7.2	7.7
	14 days		3.6	8.2
(±)-Reticuline-3-^{14}C	20 hr	31.7		1.3
	3 days	22.6		4.1
	14 days	12.6		3.2

* Plants were 128 to 139 days old and weighed from 28 to 68 g each. † No attempt was made to recover unmetabolized dehydroreticulinium chloride due to its instability during plant extractions.

Table 2. Alkaloid isolation procedures. Purifications achieved from simulated plant extracts.

A (mg)	B (mg)	Purification Sequence*	Remaining B in A
Codeinone† (100)	Codeine-^{3}H (2)	f,f	6.6×10^{-5}%
Codeine (85)	Codeinone-^{3}H (85)	c,f,f	1.0×10^{-2}%
Codeine (150)	Morphine-^{3}H (150)	a,b,e	6.2×10^{-8}%
Morphine (150)	Codeine-^{3}H (150)	a,e,e	1.7×10^{-8}%
Oripavine (27)	Thebaine-^{3}H (106)	a,d,e	1.8×10^{-4}%
Northebaine (50)	Thebaine-^{3}H (75)	d,d,d,e	6.5×10^{-3}%

* Purification steps according to letter designations are explained in Experimental. † Synthesis provided codeinone before purification with 0.8% codeine contamination.

Table 3. Thebaine metabolism. Summary of the precursors fed and their incorporations into metabolic products.

Expt. No./ Duration	No. of plants/age (mo)/wet wt. (g)	Precursor fed/ mg/dpm x 10^6	Precursor % Metabolized	Product/% Incorp. of Metabolized Precursor
1/50 hr	3/6/90	Codeinone-^{3}H/18/30.0	100	Codeine/12.0
2/7 days	3/6/110	Codeine-^{3}H/21/241	50	Morphine/0.09
3/7 days	3/6/180	* Codeine-^{3}H/32/172 Morphine/30/0 † Morphine/24/0 Morphine/33/0	67	Morphine/0.07
4/5 days	4/6/95	Thebaine-^{3}H/20/324	61	Oripavine/0.06
5/5 days	4/6/130	Thebaine-^{3}H/20/326	46	Oripavine/0.31 Northebaine/0.48
6/5 days	3/7/90	Oripavine-^{3}H/11/4.47	50	
7/5 days	3/7/80	Oripavine-^{3}H/0.12/0.051	67	
8/5 days	3/7/70	Northebaine-^{3}H/18/37.6	49	
9/5 days	3/7/80	Northebaine-^{3}H/0.13/0.38	41	

* Co-injected. † Cold morphine was injected on the third and fifth days.

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