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METABOLISM OF ^{14}C-CODEINE IN CELL CULTURES OF *PAPAVER SOMNIFERUM*

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Key Word Index—*Papaver somniferum*; Papaveraceae; metabolism; ^{14}C-codeine; cultured cells.

Abstract—Callus of *Papaver somniferum* was induced from seedling hypocotyls on Murashige and Skoog's agar medium. Cell cultures were initiated by transferring the callus to Gamborg's B5 medium containing 2,4-D. Somatic embryogenesis was induced by transferring the suspended cultured cells to 2,4-D free Gamborg's B5 medium. TLC and HPLC indicated that the extracts from the cultured cells contained thebaine as the major morphinan alkaloid and small amounts of codeine, while morphine was not detected. Addition of ^{14}C-codeine to the cultured cells demonstrated that ^{14}C-codeine was predominantly converted into *N*-oxide products along with the formation of minor amounts of morphine, indicating that induced poppy cell cultures could synthesize morphine with low efficiency.

INTRODUCTION

The capability of cell cultures of opium poppy (*Papaver somniferum*) to produce morphinan alkaloids, such as morphine, codeine and thebaine, has been a major subject of research. Several studies [1–3] have described methods of inducing maximum production of morphinan alkaloids in poppy cultures. Other findings [4–6] suggest that the physiological stage of the cultured cells determines the capability to produce alkaloids. For example, in one investigation thebaine and codeine were produced in the undifferentiated stage of cultured cells while morphine was produced in the differentiated stage [4]. Other reports [5, 6] indicate that cultured poppy cells that produce morphinan alkaloids contain large amounts of tracheal elements or redifferentiated roots. Other investigations [7, 8] suggest that either the embryogenesis or the differentiation stage of cultured cells, which produce lactifers, will generate morphinan alkaloids. The presence of lactifer cells was necessary for the formation of the alkaloids in germinating *Papaver somniferum* seedlings [8, 9]. While somatic embryogenesis could be induced in *P. somniferum* [7, 10], thebaine was the only morphinan alkaloid positively identified at the embryoid stage of cultured cells [9, 11]. Other investigators, using an enzyme immunoassay technique, [12] showed that cultured cells produced morphinan alkaloids when tracheal elements were formed. These studies indicate that cultured poppy cells have the ability to synthesize morphinan alkaloids at a stage closely related to the differentiation of some tissues and organs. However, the concentration of morphinan alkaloids formed in these cells was not as high as intact capsules. A large yield of morphinan alkaloid could be produced if the cultured cells regenerated into plants [13]. Based on the inherent problems of producing sufficient amount of morphinan alkaloids in *P. somniferum* cultures, we concentrated our study on the factors that could affect the biotransformation of codeine to morphine. In this report, we investigated the ability of codeine to convert to morphine in cultured cells of *P. somniferum* by a radioisotope technique. Results suggest that enzymes involved in the oxidative degradation of codeine dominate the biotransformation of codeine to morphine in *P. somniferum* cultured cells.

RESULTS AND DISCUSSION

Following the procedures [7, 10] for induction embrogenesis, 2,4-D was deleted from the suspension cultured cells of *P. somniferum*. Ten to 45 days after suspension cells were transferred to 2,4-D free B5 medium, the alkaloids were extracted and separated by TLC. Even after 45 days of induction, morphine was not found in extracts from the induction cultures (Fig. 1). Compounds were identified by R_f values using authentic codeine and thebaine as standards. The quantity of morphinan alkaloids formed during the induction was further determined by HPLC. The HPLC analysis of the alkaloid extracts from the induced cultured cells indicated that thebaine was the major alkaloid, while codeine appeared in moderate amounts. Morphine was not detected in the mixture by HPLC even after 60 days of induction.

In order to explore the basis of why morphine production was lacking in cultured cells at the early stage of induction, a radioactive isotope labelling technique was employed which could detect the formation of morphinan alkaloids in small quantities. ^{14}C-codeine was introduced into the cultured cells at the beginning of embryogenic induction (removal of 2,4-D from culture medium) and the labelled cells were harvested five and 51 days after induction. The extracted alkaloids were then separated by TLC. Radio-TLC of both five and 51 day cultured cells showed a radioactive zone near the origin which was identified as codeine *N*-oxide (C-NO). For the five-day

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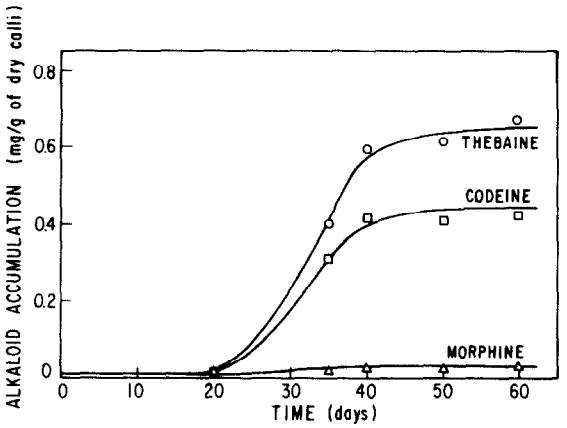

Fig. 1. Alkaloid accumulation during growth of cultured *P. somniferum* cells. After 20 to 60 days of induction, alkaloids were extracted from cultured cells and analysed by HPLC as described in Experimental.

Table 1. Distribution of radioactivity in 51-day induced cultured cells*

Alkaloids†	Migration distance (cm)	% Total radioactivity
N-Oxides	3.2	4.5
Unknown (I)	7.0	2.0
Morphine	9.0	1.2
Codeine	10.2	90.9
Unknown (II)	15.4	1.5

*Cultured cells were grown in Gamborg's B5 medium without hormone.

†Alkaloids were separated and purified as described in Experimental.

induced cells, the remainder of the radioactivity was recovered as ^{14}C-codeine, while the 51-day induced cells were able to convert ^{14}C-codeine to ^{14}C-morphine and other unidentified ^{14}C-labelled compounds. The distribution of radioactivity at the five- and 51-day inductions of various alkaloids is summarized in Table 1. At 51 days of induction, 10% of ^{14}C-codeine was metabolized to other alkaloids. Of these metabolized alkaloids, only 1% of the radioactivity was recovered as morphine as determined by mass spectral analysis. More than 4% of the radioactivity appeared as the *N*-oxide peak. Identification of morphine and the *N*-oxide was based on the comparison of TLC separated labelled compounds and mass spectral fragmentation patterns.

Inconsistent results in the literature regarding the biosynthetic capability of opium poppy cultures to produce morphinan alkaloids may be due to both the various development stages of cultured cells used for such studies and because of the differing techniques used to detect alkaloids. Data reported previously [5, 10] indicates that induced cultured cells produce thebaine and codeine, but not morphine. However, a report by Galewsky and Nessler [11] using GC-MS techniques indicated that the compound found from the induced cells was a codeine-like substance, but not authentic codeine. This compound had the same retention time and R_f value characteristic as codeine, but not the same absorptivity ratio (A_{280}/A_{254}), nor the same GC-MS analysis data. This codeine-like material is a nitrogen compound with an apparent M_r of 279 which does not correspond to the authentic M_r of codeine (299).

The above results with cultures of *Papaver somniferum* undergoing embryogenesis suggest that oxidative degradation processes limited the functioning of the biosynthetic enzyme required to convert codeine to morphine. This oxidation reaction may be a major cause of the observed low yields of morphine in the cells. Thus, further research should focus on developing methods to study the factors which will stimulate enzymes involved in the biosynthesis morphinan alkaloids in induced cultured cells. One alternative way to increase the production of morphinan alkaloids was demonstrated by Furuya *et al.* [14]. They mobilized the *P. somniferum* cells and have shown the conversion of codeinone to codeine in 20–30% yield.

Our preliminary results of SDS polyacrylamide gel electrophoresis (data not shown) indicate that certain proteins found in differentiated cells and the latex of the opium poppy were absent in the undifferentiated culture cells. One might expect that these new proteins found in the differentiation stage of cultured cells may be involved in the biosynthesis of morphinan alkaloids. However, the exact role of these proteins remains to be established.

EXPERIMENTAL

Seeds of *P. somniferum* were provided by Dr Quentin Jones (Agricultural Research Service, USDA, Beltsville, Maryland, U.S.A). Radioactive (^{14}C-methyl codeine) (sp act 56 mCi/mmol) was obtained from Amersham. Thebaine, codeine and morphine were gifts from the Penick Corporation (Lyndhurst, NJ 07071, U.S.A.). Other standard alkaloids used for MS analysis were obtained from Applied Science Laboratories. Codeine *N*-oxide and morphine *N*-oxide were synthesized according to previous published methods [15]. All other chemicals were of the highest grades available from commercial sources.

Induction of callus tissue. Seeds of *P. somniferum* were surface sterilized with 10% Clorox, then rinsed with sterilized H_2O and placed in Petri dishes. Seedlings formed after one week of germination, and the hypocotyl segments were sectioned and then placed on Murashige and Skoog's agar medium [16] containing 1 ppm of 2,4-D and 0.1 ppm of kinetin. After 4 weeks at 25° in the dark, the resulting callus was used to initiate liquid suspension cultures by transferring the callus into aqueous B5 medium of Gamborg [17] containing 1 ppm of 2,4-D.

Inoculation of suspension cultured cells. Cells (*ca* 3 g fr. wt) in the liquid culture suspension were transferred into a 200 ml Erlenmyer flask with 40 ml of Gamborg's B5 liquid medium without 2,4-D. Cultures were then grown in the dark at 25° with constant shaking (100 rpm) and subcultured every 5 days. For the ^{14}C-codeine expts, 5 μCi of ^{14}C-codeine and 1 mg of codeine were added to each flask containing 40 ml of liquid medium and ^{14}C-codeine was reintroduced during each subculture step thereafter.

Extraction and analysis of alkaloids. Cultured cells were collected by filtration and lyophilized for 24 hr. The alkaloids in the dry cells were then extracted [18] with $CHCl_3$–*i*PrOH (3/1). The dried residue was dissolved in 0.5 ml EtOH. Aliquots of 50 μl were spotted on TLC plates (Silica G, 250 μm) and developed in Me_2CO–xylene–MeOH–NH_4OH (89:83:10:8). Radioisotope

labelled alkaloids, TLC plates were scanned with a linear analyzer which automatically integrates the radioactivity of labelled compounds. After scanning, the plate was sprayed with Dragendroff's reagent [19] to locate morphinan alkaloids. Data presented in this study are the average of duplicate experiments.

HPLC analysis of alkaloid extracts. A column (30 cm × 3.9 mm i.d.) packed with 5 μ porous silica gel was used with a multi-wavelength UV-visible detector and a syringe-loading injector with 100 μl loop. The solvent used was *n*-hexane–$CHCl_3$–EtOH.–Et_2NH (60:6:8:0.1). Conditions were column temp. 25°; flow rate, 2 ml/min; detector wavelength 285 nm.

MS analysis of extracted alkaloids was carried out after prep. TLC as described in a previous report [3].

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