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BIOTRANSFORMATION OF THEBAINE BY CELL CULTURES OF *PAPAVER SOMNIFERUM* AND *MAHONIA NERVOSA**

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Key Word Index—*Papaver somniferum*; Papaveraceae; *Mahonia nervosa*; Berberidaceae; thebaine; codeine; tetrahydrothebaine; thebainone; oripavine.

Abstract—A cell suspension culture of *Papaver somniferum* that accumulates small amounts of thebaine was used to investigate the metabolic fate of exogenously supplied [N-$^{14}CH_3$]thebaine. Three metabolites of thebaine still containing the label were discovered and identified as codeine and two new metabolites, tetrahydrothebaine and thebainone. This proves that the poppy cell culture contains the enzymes necessary for enolether cleavage of thebaine at carbon atom 6. Screening of 60 cell suspension cultures of different plant species and of 230 field grown plant species for their capability to metabolize [N-$^{14}CH_3$]thebaine showed that this compound is metabolically inert and only the single cell culture of *Mahonia nervosa* was capable of cleaving the phenolether methyl group at carbon atom 3 of thebaine to yield oripavine. The field-grown plant of *Mahonia nervosa* in contrast could not perform this reaction. © 1997 Elsevier Science Ltd

INTRODUCTION

Up to now not a single cell culture of *P. somniferum* has been found that is reliably producing the (*R*)-configured opium alkaloids codeine and morphine. All reports so far published have to be viewed with scepticism. The reason may be that *Papaver* cell cultures lack the ability to form latex vesicles in culture, a condition that is believed to be essential for the accumulation of these alkaloids [1]. Cell cultures of *Papaver*, however, are also reported to lack the enzymes [2] for funneling thebaine, an important branch point intermediate either into the codeine or into the oripavine branch of morphine biosynthesis (Fig. 1). On the other hand, cell and callus cultures of opium poppy quantitatively convert (*S*)-reticuline into (*S*)-configurated alkaloids of the benzophenanthridine and aporphine type, so that this ability can be even used to produce optically pure (*R*)-reticuline from racemates of this alkaloid [2]. This indicates that the enzymes that demethylate thebaine either to the neopinone/codeinone pair [3] or to oripavine [4] are not expressed in the dedifferentiated cellular state.

The pathway from tyrosine to thebaine has been clarified in precise details at the enzyme level in *P. somniferum* [5 and literature cited]. In our attempt to completely unravel the morphine pathway in this plant, we investigated the metabolic fate of thebaine in a poppy cell culture, in order to gain insight into the nature of the enzymes possibly involved in the transformation of this intermediate. For this study we used a cell culture that was selected by a radioimmunoassay screening of 333 different cell suspension cultures derived from 333 different seed samples collected the world over for their ability to produce morphine alkaloids [6]. Only a single culture was found consistently synthesizing thebaine at an average level of 7.5 mg l^{-1} culture medium in a growth period of nine days for more than 10 years [7]. We report here on a transformation of radioactively labelled thebaine into codeine, thebainone and tetrahydrothebaine by this cell culture.

RESULTS AND DISCUSSION

In order to follow the metabolism of thebaine in the completely dedifferentiated suspension culture of *P. somniferum*, [N-$^{14}CH_3$]thebaine was chosen. The formation of all commercially interesting opium alkaloids, all containing an [N-CH_3] group, could be followed, as well as the possible formation of *N*-oxides [8]; *N*-demethylation of thebaine or further metabolites, however, could not be detected since the label would be lost and disappear in the background radioactivity of that culture.

[N-$^{14}CH_3$]Thebaine of high specific activity (60

* Dedicated to Prof. W. Beck, Nünchen, on the occasion of his 65th birthday.

† Author to whom correspondence should be addressed.

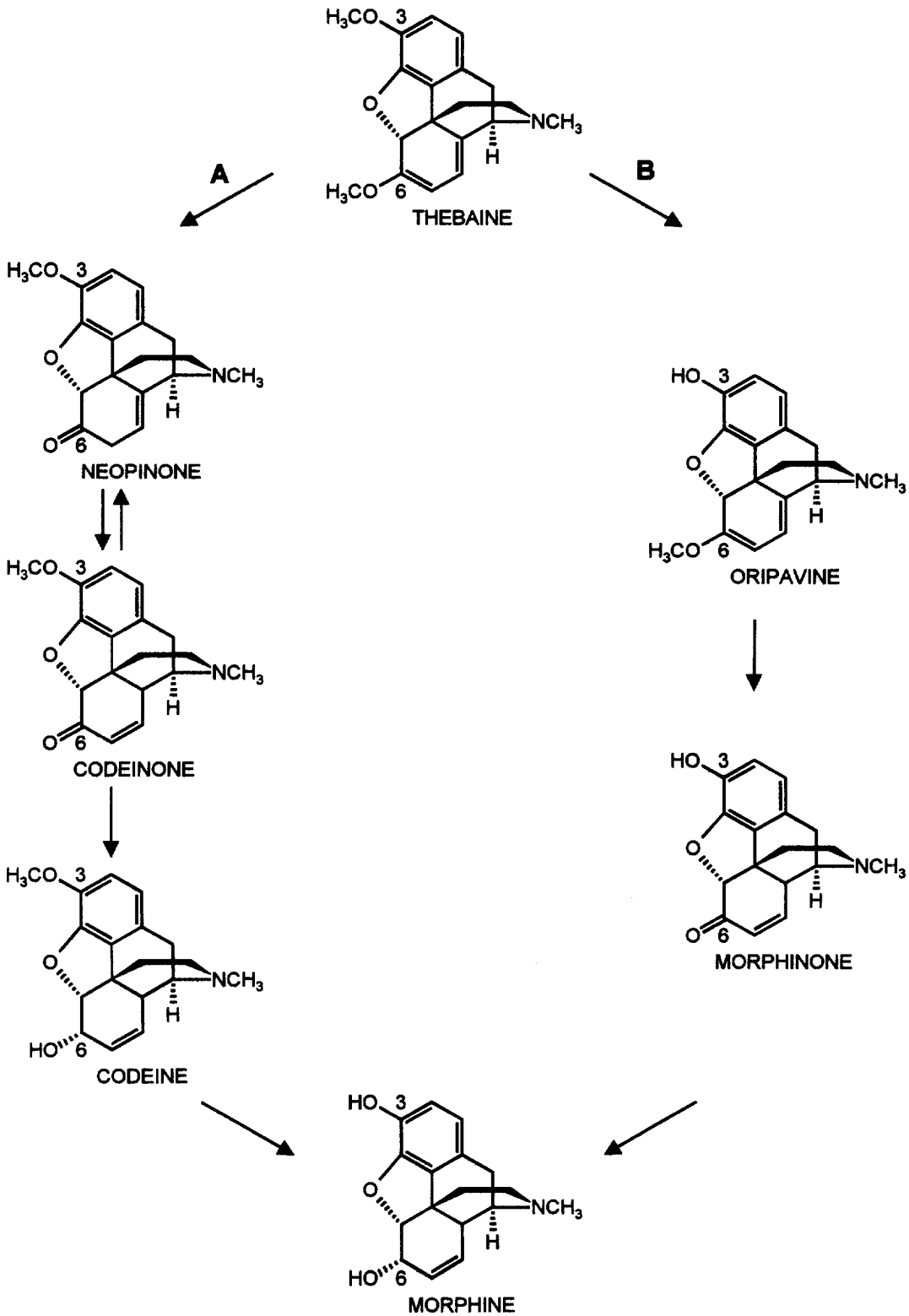

Fig. 1. Biosynthetic conversion of thebaine to morphine by two possible pathways A and B. Each pathway requires the demethylation of thebaine. While route A leads to morphine via codeine after enolether cleavage of thebaine, the alternative route B involves oripavine as a metabolite resulting from 3-*O*-demethylation of thebaine.

μCi · μmol^{-1}; 3.3 nmol) was added to a cell suspension culture in a small volume (1.5 ml; 0.5 g fr. wt) under constant agitation. After 5 days of incubation, the tissue was extracted (ethanol) and subjected to TLC (solvent system I). As shown in Fig. 2, all of the radioactive thebaine taken up by the cells was metabolized and two main metabolites resulted, one with an R_f of 0.34 containing 31% of the total radioactivity of the extract, the other with an R_f of 0.56 containing 26% of radioactivity. No radioactivity was seen at an R_f value of morphine. The two main metabolite-containing zones were eluted and rechromatographed in solvent system II. The peak with the lower R_f value split under these conditions into two metabolites clearly distinct with an R_f of 0.43 (metabolite A) and R_f of 0.59 (metabolite B). The compound with the higher R_f in solvent system I was found to be a single compound upon rechromatography (metabolite C). Metabolites A–C were rechromatographed in solvent system III and no additional radioactive zones appeared. While metabolite A consistently had the same R_f as codeine in all three solvent systems used, metabolites B and C did not co-chromatograph with any known metabolites of the morphine pathway. In order to investigate whether any of these three metabolites could be regarded as a precursor of morphine, the isolated radioactive compounds were fed independently to 26-day-old seedlings of *P. somniferum*, known to produce at that stage labelled morphine from all potential labelled precursors of the morphine pathway. This test gave a clear result: metabolite A was transformed into morphine while metabolites B and C were not. Metabolite A, therefore, had to be regarded as a potential precursor of morphine, while metabolites B and C were possible degradation products not located in the main morphine pathway. In order to exclude the possibility that the metabolites B and C were *N*-oxides, these metabolites were independently treated with 6% H_2SO_3 known to reduce alkaloidal *N*-oxides [8]. This treatment did not affect the polarity of the metabolites under investigation.

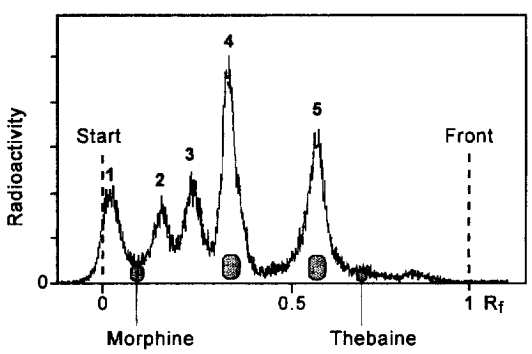

Fig. 2. Radio-TLC of an EtOH-extract of *Papaver somniferum* cells after an incubation period of 5 days with carrier-free [N-^{14}CH$_3$]thebaine. Two main metabolites (peak 4 and 5) could be detected, one with an R_f value of 0.34 (peak 4) the other with an R_f value of 0.56 (peak 5). The precursor thebaine was totally metabolized.

Control experiments using this procedure with authentic radioactive *N*-oxides of thebaine, codeine and morphine generated by a pig liver enzyme preparation resulted in a quantitative reduction of these quaternary alkaloids [H. Huh, T. Amann, M. H. Zenk, unpublished]. Therefore, it could be excluded that B and C are *N*-oxides. Since metabolite A was suspected, due to its chromatographic behaviour, to be similar to codeine, this radioactive metabolite was subjected to the action of codeinone reductase isolated from *P. somniferum*, which is a freely reversible enzyme [9]. On incubation with the homogenous enzyme in the presence of NADP, the radioactive metabolite was transformed into a compound that was indistinguishable from codeinone, both by TLC and HPLC [3]. This was a strong indication that metabolite A is indeed codeine.

Since metabolite C upon chromatography in all three TLC solvent systems travelled identically or close to an authentic codeinone/neopinone mixture, an attempt was made to reduce this metabolite by BH_4^-. However, the chromatographic behaviour of this compound was not changed, which excluded the presence of a keto group in that molecule. Since the experiments up to that point were conducted with carrier-free radioactive thebaine metabolites in the ng-range, this clearly did not allow a structural identification of these metabolites. In order to obtain substantially larger amounts of the three main thebaine metabolites, the production condition of the suspension cultures had to be optimized. 100 ml conical flasks were incubated for a period of 4 days containing within 25 ml *P. somniferum* cell culture a constant 0.2 μCi (3.4 nmol) [N-^{14}CH$_3$]thebaine and increasing amounts of unlabelled thebaine. Table 1 shows the result of this experiment. While all flasks showed a final average dry weight of about 1.1 g, thebaine showed no toxic effect even at 1 mM concentration as measured by growth compared with a control where no unlabelled thebaine was added. Thebaine uptake was relatively constant between 30 and 55%. The formation of metabolite A increased with concentration of unlabelled thebaine up to 100 μM. Interestingly, 1 mM thebaine did not affect growth but completely suppressed its transformation, possibly by 'feed forward' inhibition. This fact should be taken as a note of caution not to overload the metabolic systems of cell suspension cultures by adding concentrations of potential precursors into the growth or production media which are excessive. In order not to negatively affect the transformation capabilities of the cell culture system and to obtain reproducible results, it was decided to use only 7 μM thebaine for the planned biotransformation studies. With that concentration, first a time course of the transformation rate for thebaine was studied (Fig. 3). Clearly the highest stationary concentration of thebaine transformation under these conditions was found on day 6, which was used subsequently for the isolation of the three thebaine metabolites. Having thus optimized the

Table 1. Feeding experiment to optimize the production conditions of the *Papaver somniferum* cell culture. The transformation capability of the cell culture could be increased up to a thebaine concentration of 100 μM. For further biotransformation studies, a thebaine concentration of 7 μM was used (n.d. = not detectable).

Assay	1	2	3	4	5	6	7	8
Labelled thebaine [μCi]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Unlabelled thebaine [μM]	0	0.2	1	3	10	30	100	1000
Fresh weight [g]	9.2	11.2	13.1	13.5	13.0	8.9	12.8	10.6
Thebaine uptake [%]	32	38	21	20	24	32	57	45
Metabolite A [nmol]	0.22	0.38	0.95	2.66	9.0	22.3	63.0	n.d.
Metabolites B+C and others [nmol]	0.43	0.74	2.14	6.38	26.5	86.7	107.3	n.d.

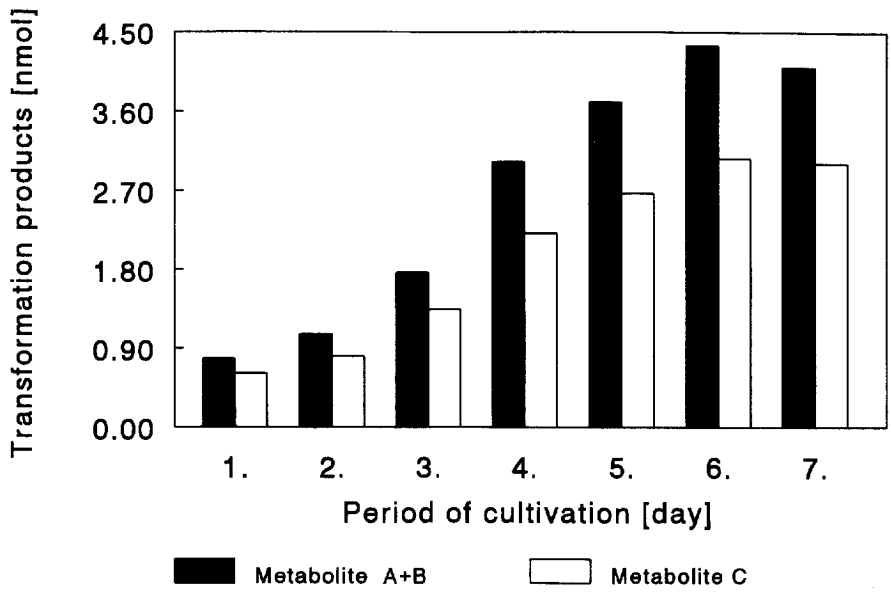

Fig. 3. Kinetic study of the transformation of thebaine by *Papaver somniferum* cell cultures. A total of 1.5 ml of medium containing 100 mg (dry weight) of cells were cultivated in the presence of 7 μM thebaine containing 0.1 μCi [N-$^{14}CH_3$]thebaine for a period of up to one week. The cells were analysed after different times for their contents of metabolites A + B and C by TLC.

transformation conditions of the poppy cell culture towards thebaine, a large scale incubation of thebaine with the poppy culture was set up. Four one litre conical flasks, each containing 400 ml of a 3 day cell culture and 7 μM thebaine, were incubated for 6 days. In parallel, four 100 ml conical flasks, each containing 25 ml cell suspension culture with 0.8 μCi [N-$^{14}CH_3$] thebaine, were incubated under the same conditions as above. The cells from both incubations were combined, harvested and extracted with ethanol. The ethanol was largely removed and the aqueous residue was made alkaline (pH 9.5) and thoroughly extracted with chloroform. The organic phase was dried and evaporated. The thebaine metabolites were isolated by TLC, the metabolites were located by their radioactivity, eluted and subsequently rechromatographed in two different additional TLC systems (IV and V). By this procedure, starting from a total of 3.5 mg thebaine, 76 μg metabolite A, 11 μg of metabolite B and 35 μg of metabolite C were obtained in pure form. Prior to mass spectroscopic analysis, the metabolites were subjected to solid phase extraction (bond-elute columns). The columns with the individually absorbed metabolites (at pH 4) were washed with methanol and the metabolites were desorbed with methanol containing NH_3. The recovery of the metabolites was essentially quantitative, the eluate was taken to dryness and the metabolites subjected to mass spectrometry both in CI and EI mode. Metabolite A showed a mass $(M+H)^+$ of 300 and 282, the EI-MS showed *m/z* (rel. int.): 299 (100, M^+), 282 (4.7), 242 (4.7), 229 (13.4), 214 (8.2), 188 (4.7), 162 (21.5), 124 (11.3). Reference codeine showed an identical fragmentation pattern. Treatment of metabolite A with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide gave a trimethylsilyl derivative, that upon GC-MS showed three characteristic fragments at *m/z* 371, 343 and 234 again in absolute agreement with literature data for trimethylsilyl codeine [11]. All data obtained on this metabolite together (see above) showed that metabolite A is codeine.

Metabolite B showed in EI mode *m/z* (rel. int.): 299

(100, M^+), 284 (18.2), 256 (6.9), 242 (7.8). A molecular peak in CI-MS mode $(M+H)^+$ was 300. The mass fragmentation spectrum was identical to thebainone [10]. This metabolite is not a precursor of morphine, and as shown above, no incorporation of metabolite B into morphine could be found. Metabolite C showed in CI-MS mode $(M+H)^+$ of 316 and 298, in EI-MS *m/z* (rel. int.): 315 (100, M^+), 298 (6.5), 286 (6.5), 262 (8.5), 231 (10.0), 229 (18.8). The CI-spectrum showed that the metabolite C was heavier by four mass units than thebaine. Metabolite C showed in addition a characteristic fragment at 298. Its chromatographic, chemical and enzymatic properties and the fact that metabolite C was also not incorporated into morphine *in vivo* suggested that this alkaloid might be tetrahydrothebaine. This assumption was verified through one of the main fragments seen in EI-MS mode at 229. This characteristic fragment is formed as a positive radical from the parent compound by loss of ring C ($C_5H_{10}O_2$, which is typical for the saturated ring structure) [12]. Metabolite C is, therefore, tetrahydrothebaine.

As shown by the occurrence of codeine and thebainone as major thebaine metabolites in cell suspension cultures of *P. somniferum*, it is clear that this undifferentiated cell culture has the enzymatic capability to demethylate thebaine in position 6 (Fig. 4). This poppy cell culture, therefore, could potentially be a good biological source to discover the enolether cleavage enzyme that is of major importance and mechanistically highly interesting in the biosynthesis of codeine and morphine [13].

In order to gain more insight into the metabolism of thebaine in the plant kingdom and possibly to discover another plant species that could be used as a potential source to study *in vivo* and *in vitro* the demethylation of thebaine or other transformation reactions of this opium alkaloid, carrier-free [N-$^{14}CH_3$]thebaine was incubated with cell cultures of a total of sixty different plant species. After incubation for a period of 5 days, the cells were extracted and the extract analysed for potential metabolites by TLC. The following 59 species from 14 plant families were tested and absolutely no transformation of thebaine was detected: Taxaceae: *Taxus baccata*; Monimiaceae: *Peumus boldus*; Ranunculaceae; *Coptis japonica*, *Thalictrum glaucum*, *Thalictrum tuberosum* yellow, *Thalictrum tuberosum* white; Berberidaceae: *Berberis aggregata*, *Berberis aggregata* var. *prattii*, *Berberis aristata*, *Berberis beaniana*, *Berberis canadensis*, *Berberis candidula*, *Berberis carminea*, *Berberis concimma*, *Berberis concinata*, *Berberis crataegina*, *Berberis darwinii*, *Berberis dictyophylla*, *Berberis frauchetiana*, *Berberis geraldii*, *Berberis hanniensis*, *Berberis heuryana*, *Berberis julianae*, *Berberis koetiana*, *Berberis lycium*, *Berberis mucrifolia*, *Berberis nobilis*, *Berberis nummularia*, *Berberis papillifera*, *Berberis somnifolia*, *Berberis stolonifera*, *Berberis vulgaris*, *Mahonia nervosa*; Papaveraceae: *Chelidonium majus*, *Corydalis vaginans*, *Corydalis thalictrifolia*, *Eschscholtzia californica*, *Papaver bracteatum*, *Papaver nudicaule*; Caryophyllyceae: *Silene cucubalus*; Chenopodiaceae: *Beta vulgaris*; Fabaceae: *Crotalaria cobalticola*, *Erythrina crista-galli*, *Phaseolus vulgaris*; Rutaceae: *Citrus decuminata*; Apiaceae: *Petroselinum crispum*; Malvaceae: *Gossypium herbaceum*; Rubiaceae: *Rubia tinctorum* Sally; Apocynaceae: *Catharanthus roseus*, *Rauwolfia chinensis*, *Rauwolfia verticullata*; Solanaceae: *Lycopersicon esculentum*, *Nicotiana alata*, *Nicotiana tabacum*, *Solanum marginatum*; Convolvulaceae: *Ipomea alpina*; Scrophulariaceae: *Mimulus guttatus*; Dioscoreaceae: *Dioscorea composita*; Liliaceae: *Muscari botyroides*; Poaceae: *Agrostis tenuis*.

As seen from these experiments, thebaine is surprisingly metabolically inert and other plant species aside from *P. somniferum* are unable to either demethylate this compound or to hydrogenate ring C. There was, however, a single exception. The yellow pigmented cell culture of *Mahonia nervosa* Nutt. that produces abundant amounts of protoberberine alkaloids like other members of the family Berberidaceae, transformed thebaine under the above conditions to an unknown metabolite in good yield (35%). This new metabolite was not identical on TLC comparison with the metabolites A, B or C. The *Mahonia* culture produced only this single metabolite D. Larger scale incubation of unlabelled thebaine with *M. nervosa* cells and isolation and purification of the single metabolite D in exactly the same manner as above yielded 101 μg of the unknown. CI-MS $(M+H]^+$ of this compound yielded a molecular mass of 354. EI-MS mode *m/z* (rel. int.) yielded fragments at 297 (100, M^+), 282 (11.5), 266 (11), 254 (20.5), 241 (22), 223 (13), 211 (5). The spectrum of metabolite D was identical in all respect to the EI-MS spectrum of reference oripavine [14]. Biological proof was also given for the correct structure of metabolite D when feeding of the radioactive compound, isolated after transformation of labelled thebaine through *M. nervosa* cell culture, to *P. somniferum* seedlings gave a 13% conversion into morphine. No other metabolites were detected besides morphine. The exclusive transformation of oripavine into morphine by opium poppy plants had been observed before [4]. Only the cell culture of *M. nervosa* was capable of converting thebaine into oripavine (Fig. 4). None of the closely related 26 different species of *Berberis* tested transformed thebaine. Much to our surprise, not even differentiated shoots of outdoor grown *M. nervosa*, the identical plant from which the cell culture had been established, was able to conduct the phenolether cleavage at position 3 of thebaine to oripavine. No conversion of thebaine to oripavine or any other thebaine metabolite still containing the N-CH_3 group was observed when the following *Mahonia* species (shoots) were supplied with radioactive thebaine: *Mahonia aquifolium*, *M. bealei*, *M. japonica*, *M. patagonica*. As a result of this inability to chemically modify thebaine by differentiated plants, a total of 230 differentiated plant species representing 94 different lower and higher plant families were analysed (list

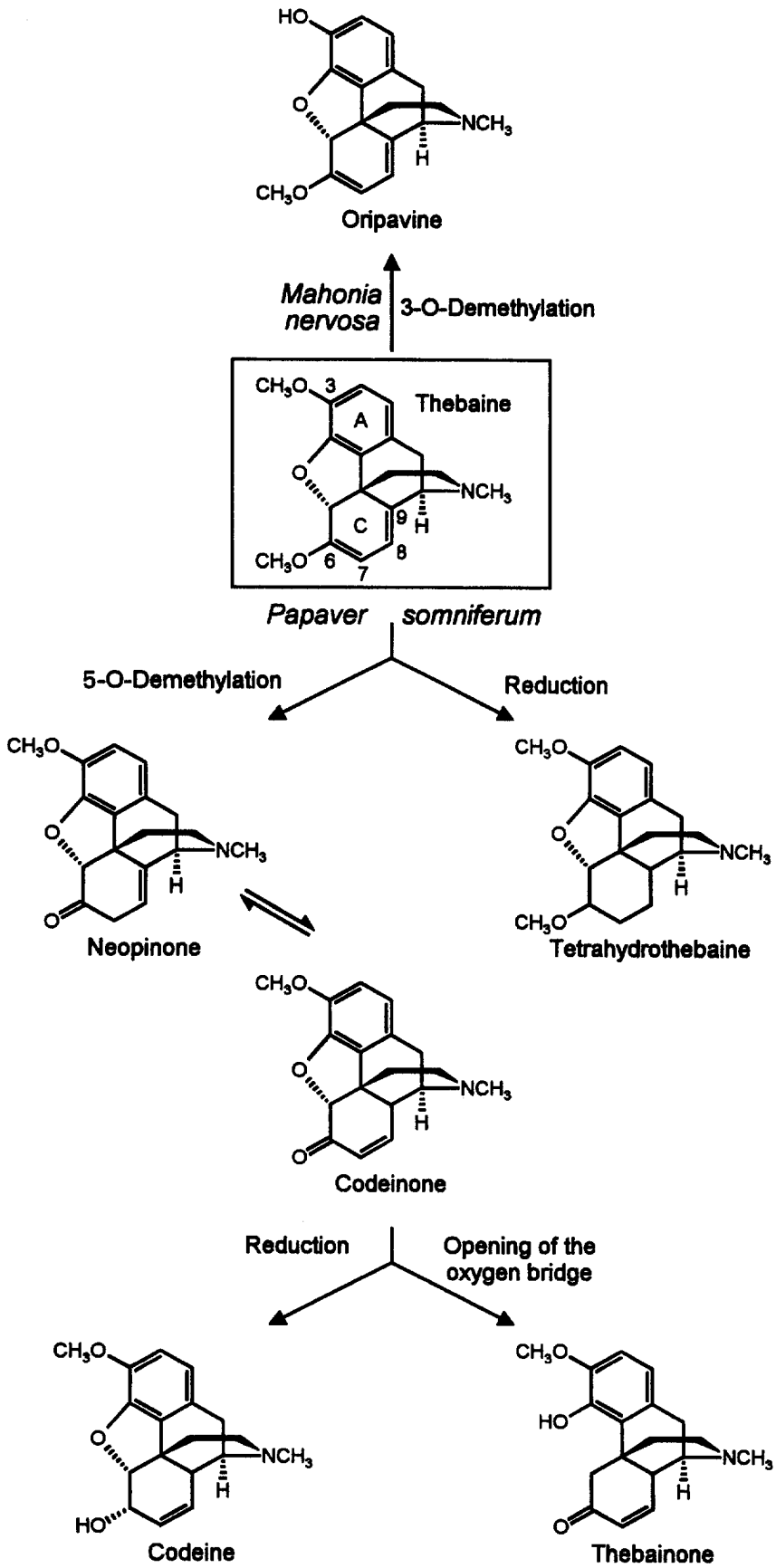

Fig. 4. Biotransformation of thebaine by cell cultures of *Papaver somniferum* and *Mahonia nervosa*. Three metabolites of thebaine were isolated from the poppy cell culture and identified as codeine, tetrahydrothebaine and thebainone. *Mahonia nervosa* was capable of cleaving the phenolether methyl group of thebaine to yield oripavine.

available on request). The only field grown species that showed any metabolism of thebaine was *P. somniferum* that showed a 41% conversion of thebaine under these conditions.

The fact that dedifferentiated cell cultures of *M. nervosa* have the enzyme system to demethylate thebaine by phenolether cleavage to oripavine but not differentiated plants of the same species may indicate, that under cell culture conditions genes are activated that in the differentiated stage of the plant are silenced. Curiously enough, radioactive codeine when supplied to the *M. nervosa* cell culture was not converted to morphine, which would have been the equivalent reaction, namely 3-*O*-demethylation, that this plant cell culture is capable of effecting with thebaine. This shows an extreme case of specificity for the 3-*O*-demethylating enzyme, a specificity not shown by the opium poppy plant [4]. In general, thebaine is an extremely metabolically inert compound, which out of 230 different plants so far tested is only converted by two species to metabolites. Whole plants and cell cultures of *P. somniferum* where this compound is a proven intermediate in the morphine biosynthetic pathway could be expected, but transformation by *M. nervosa* cell cultures, but not by the differentiated plant is difficult to explain. In the latter case not even a clue as to an evolutionary trend can be given, since *Mahonia* species and derived cell cultures all produce the same isoquinoline alkaloids and no compounds containing the morphinan skeleton with (*R*)-configuration are known to occur in this genus.

The present study demonstrates that thebaine is an exceedingly metabolically inert alkaloid, only highly specialised morphine and codeine containing *Papaver* species [6] are capable of conversion of the enolether of thebaine to the keto group of neopinone and thereby to codeinone [13, 3]. The enzyme catalysing this reaction in the opium poppy is supposed to be a cytochrome P-450 enzyme. It could be shown that the *P. somniferum* cell suspension culture transforms thebaine both to codeine and thebainone, the latter may arise by enzymatic opening of the ether bridge at the stage of codeinone. The third metabolite found in the poppy cell culture must arise from a specific reduction of the 6, 7 and 8, 9 double bonds of thebaine (Fig. 4) as has been similarly observed for the reduction of morphinone/codeinone by a bacterial morphinone reductase [15]. The fact that this enzyme is present in the *Papaver* cell culture should make possible a biochemical investigation as to the nature of this enzyme. Differentiated field grown *P. somniferum* plants, most likely because of their high phenolic content, have made the detection of this enzyme system impossible up to now. It has clearly been shown by Brochmann-Hanssen [4] that in opium poppy a second pathway exists to morphine via oripavine. This implies a cleavage of the phenolic *O*-methyl group at C-3 of thebaine. This enzyme is also believed to be a cytochrome P-450 enzyme by analogy to mammalian liver enzymes known to catalyse this reaction [16, 17].

EXPERIMENTAL

Biological material. All cell cultures were provided by the departmental cell culture laboratory. The cultures were grown in LS medium [18] at 23° under constant illumination (650 lx) at 100 rpm. Field-grown plants were provided by the Botanical Garden, Munich.

Substrates. Thebaine was purchased from SANOFI, France. Codeine and morphine were from Sigma. [N-$^{14}CH_3$]Thebaine was synthesized from northebaine and $^{14}CH_3I$ (J. Gollwitzer and M. H. Zenk, unpublished). The compound had a sp. act. of 60 mCi $mmol^{-1}$.

Application. If not otherwise stated, 0.2–0.5 μCi of the labelled substrate was added carrier-free to a 24 well multiwell plate containing 1.5 ml medium [18] and about 0.05–0.1 g cells (dry wt) of a 3-day-old cell suspension culture. The multiwell plate was sealed with parafilm and put on a reciprocal shaker with 140 strokes per min. Larger scale incubations were done in 100 ml conical flasks containing 25 ml or in 1 liter flasks containing 400 ml medium. Differentiated plant material, single leaves or shoots (between 0.037 and 1.25 g fr. wt), were placed into 300 μl application soln containing 0.2 μCi [N-$^{14}CH_3$]thebaine. Incubation was done in a growth chamber at 25°C, 30–35% rel. humidity and constant illumination at 7000 lx for 24 hr. The soln was taken up and the feeding soln replaced with H_2O. In either case the tissue was extracted in 20 ml boiling 80% EtOH for 15 min under reflux, the organic phase was evapd, the residue taken up in 0.5 ml 80% EtOH, the radioactivity taken up determined in that concentrate and subjected to TLC.

Analytical methods. The following solvent systems for TLC (Polygram SIL G/UV_{254}) were used. For isolation of metabolites and for the determination of radioactive metabolites, 5 solvent systems were used: I: toluene–EtOAc–Et_2NH = 7:2:1; II: CH_2Cl_2–MeOH–NH_4OH (25%) = 90:10:1; III: EtOAc–MeOH–NH_4OH (25%) = 17:2:1; IV: toluene–Me_2CO–EtOH–NH_4OH (25%) = 45:45:7:3; and V: $CHCl_3$–EtOH–EtOAc–Me_2CO–NH_4OH (25%) = 6:2:2:1:0.5 were used. The HPLC system used was the same as published previously [3].

Alkaloid extraction. The cells were sepd from the medium by centrifugation and extracted in 20 ml boiling 80% EtOH under reflux. The tissue extract was filtrated and the resulting filtrate was concd to a final vol. of 0.5 ml.

Mass spectrometry. Performed with a Finnigan MAT SSQ 700 instrument coupled to a gas chromatograph 3400 (Varian). Analysis was done both by GC/MS and by direct insertion mode. CI was at 70 eV with isobutane as reactant gas and EI ionization was done without reactant gas at 70 eV.

Chemical reactions. The determinations for *N*-oxides were done as follows: 0.1 μCi of each metabolite dissolved in 50 μl H_2O was mixed with 50 μl 6% H_2SO_3. After 15 min the soln was made alkaline with

1 M Na_2CO_3–$NaHCO_3$, pH 9, to stop the reaction. The alkaloids were extracted with $CHCl_3$ and the organic extract was analysed by TLC.

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