Urinary excretion of morphine and biosynthetic precursors in mice

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It has been firmly established that humans excrete a small but steady amount of the isoquinoline alkaloid morphine in their urine. It is unclear whether it is of dietary or endogenous origin. There is no doubt that a simple isoquinoline alkaloid, tetrahydropapaveroline (THP), is found in human and rodent brain as well as in human urine. This suggests a potential biogenetic relationship between both alkaloids. Unlabeled THP or [1; 3; 4-D₃]-THP was injected intraperitoneally into mice and the urine was analyzed. This potential precursor was extensively metabolized (96%). Among the metabolites found was the phenol-coupled product salutaridine, the known morphine precursor in the opium poppy plant. Synthetic [7D]-salutaridinol, the biosynthetic reduction product of salutaridine, injected intraperitoneally into live animals led to the formation of [7D]-thebaine, which was excreted in urine. [N-CD₃]-thebaine was also administered and yielded [N-CD₃]-morphine and the congeners [N-CD₃]-codeine and [N-CD₃]-oripavine in urine. These results show for the first time that live animals have the biosynthetic capability to convert a normal constituent of rodents, THP, to morphine. Morphine and its precursors are normally not found in tissues or organs, presumably due to metabolic breakdown. Hence, only that portion of the isoquinoline alkaloids excreted in urine unmetabolized can be detected. Analysis of urine by high resolution-mass spectrometry proved to be a powerful method for tracking endogenous morphine and its biosynthetic precursors.

endogenous morphine | mammalian mophine | morphine biosynthesis | stable-isotope labeled morphine | high resolution-mass spectrometry

The occurrence of endogenous morphine in mammals has been a controversy over the past three decades. Goldstein (1–3) and Spector (4–7) and their associates pioneered the assumption that morphine occurs in mammals and plays an unknown role. Other groups claimed that the morphine found in rodents is of dietary origin (8, 9). Clear answers concerning the presence of morphine in animals have been obscured by inadequate experimentation and possible laboratory contamination with morphine. Only recently, several reports appeared using sterile human cell cultures and isolated organs, which added to a body of evidence that morphine indeed occurs in mammals and human cell cultures (10–12). To substantiate these findings, we made use of the fact that morphine is excreted in urine of humans (13–15) and rats (7). The presence of morphine in urine has been unequivocally demonstrated by mass spectrometry (15), but the origin of the excreted morphine has not yet been clarified. If these trace amounts of morphine in urine are formed de novo (and are not from dietary intake), a biosynthetic apparatus should be present in mammals that catalyzes this multistep pathway.

The goal of this investigation is to demonstrate the de novo formation of benzylisoquinoline alkaloids, mainly of the morphinan type, in live rodents. Our experimental strategy involves administration of stable-isotope labeled and unlabeled distant precursors to mice followed by analysis of mouse urine by mass spectrometry. In the past, the occurrence of the simple alkaloid tetrahydropapaveroline (THP) (synonym: norlaudanosoline) has been reported in human and rodent brain tissue as well as in urine (13, 16–22). This firmly established occurrence led in the past to speculations that THP might be the precursor of endogenous morphine. Experiments were not, however, conducted to test this hypothesis. We investigate here the formation of mammalian morphine by analyzing the biosynthetic pathway at three points: first, the simple substituted metabolites formed by injection i.p. of THP; second, the phenol-coupled products formed from reticuline, a 6-O-, 4′-O-, N-trimethylated tetrahydrobenzylisoquinoline; and third, the search for morphinan compounds formed after phenol-coupling. As had been postulated by Barton and Cohen (23), the key intermediate in morphine biosynthesis is the *para-ortho* phenol-coupled product of (R)-reticuline. This key step has recently been shown (24) to be catalyzed by human cytochrome P450-dependent enzymes, providing evidence for the hypothesis that morphine can be formed in mammals. Morphine should then be formed from a pentacyclic intermediate such as thebaine. We assume that biosynthetic intermediates will be excreted in the urine of the experimental animals. These intermediates should be stable in mammalian urine, excluded from further metabolism, and should reveal the intermediates of morphine biosynthesis.

Herein we show that THP is the first alkaloidal precursor for morphine in mammals. The biosynthesis of morphine then proceeds via (R)-reticuline to salutaridine, salutaridinol and thebaine, all of which were found in mice urine after injection of relevant precursors. Thebaine injection into mouse yielded codeine, oripavine, and morphine. This indicates the presence of the bifurcate pathway found previously in plant morphine biosynthesis (25). Taken together, these results provide evidence for the de novo synthesis of morphine from THP in mammals.

Results

Transformation of THP to Salutaridine. It has been shown that human neuroblastoma cell cultures (SH-SY5Y) transform L-dopa into simple tetrahydrobenzylisoquinoline and morphinan alkaloids (11). This property of the neuroblastoma cell culture is not expressed in the differentiated live mouse. Injection (i.p.) of L-dopa labeled at either [ring-, 2-¹³C₇] or [ring-¹³C₆] with or without the DOPA-decarboxylase inhibitor benserazide did not show any incorporation into alkaloids produced by the mouse. Measurements of extracts of brain, liver, kidney [20, 40, 60, 90, 120, 180, and 240 min after injection (i.p.)] and of urine [24 h after injection (i.p.)] were made by high resolution-liquid chromatography-mass spectrometry (HR-LC-MS) with a limit of detection of 2 pmol/g
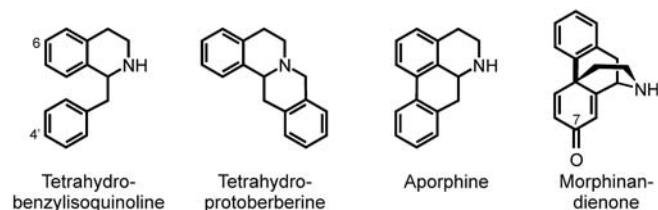
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Fig. 1. Types of alkaloids that were found excreted in the urine of mice injected (i.p.) with (*R, S*)-THP.

tissue for morphine. Neither alkaloids nor even trace amounts of the original labeled L-dopa were found. It is concluded that L-dopa was rapidly and completely degraded by the live animal to compounds that escape detection by the most sensitive routine MS instrument.

The occurrence of the tetrahydroxylated tetrahydrobenzylisoquinoline alkaloid THP in brain tissue has been convincingly reported (17–21). The regiochemistry of THP with four phenolic hydroxyl groups suggests a precursory relationship to morphine alkaloids. Indeed this potential role as precursor for morphine had already been suggested (13, 17, 21, 23, 26–28), but never experimentally tested. This attractive hypothesis was investigated to see whether THP is transformed by live mice into a phenol-coupled morphine precursor.

Injections (i.p.) of an aqueous solution of 6.1 µmol (*R, S*)-THP or [1, 3, 4-D_3]-THP into a single mouse were carried out over a period of 4 d. HR-LC-MS analysis revealed that only 30% of the injected (i.p.) alkaloid, (*R, S*)-THP, was recovered of which the major portion (96%) was identified as metabolized THP whereas a minor portion (4%) was excreted as unchanged THP. Administration of (*R, S*)-THP yielded a total of 21 distinct metabolites excreted into urine. HR-LC-MS analysis of the mouse urine made it possible to determine the alkaloidal categories excreted: tetrahydrobenzylisoquinoline, tetrahydroprotoberberine, aporphine, and morphinandienone alkaloids (Fig. 1). These four types of alkaloids were quantitatively and qualitatively analyzed (Table 1).

The fraction with the molecular mass m/z 328 deserves particular attention. The key intermediate in morphine biosynthesis, salutaridine, among other alkaloids, has this molecular mass. Careful analysis of the mixture of alkaloids by HR-LC-MS containing the molecular mass of interest showed the presence of salutaridine/sinoacutine [*para-ortho* phenol-coupled product from (*R*)- and (*S*)-reticuline, respectively, 0.01% of the total recovered alkaloids] with the fragment ions m/z 297 and m/z 265 from m/z 328 (Fig. 2*A* and *B*). In addition, [1, 3, 4-D_3]-salutaridine/sinoacutine with an experimental mass m/z 331.17298 and a deviation from the theoretical value of −0.6 ppm was detected in urine of mice injected (i.p.) with (*R, S*)-[1, 3, 4-D_3]-THP (Fig. 2 *C* and *D*).

The other six phenol-coupled products from (*R, S*)-reticuline, (+)- and (−)-pallidine (*para-para* coupling, 0.08%, retention time 11.5 min), (−)- and (+)-corytuberine (*ortho-ortho* coupling,

0.01%, retention time 14.9 min) and (−)- and (+)-isoboldine (*ortho-para* coupling, 0.07%, retention time 17.1 min), could be isolated and identified in addition to salutaridine/sinoacutine. The finding of a set of eight phenol-coupled products after (*R, S*)-THP administration was identical to that of the enzymatically generated phenol-coupled products as described by (24). The above results, however, verify for the first time the formation of the alkaloidal phenol-coupled products in mice. In addition to the phenol-coupled products, two of the four dimethoxy-tetrahydroprotoberberine alkaloids were identified as coreximine (molecular mass m/z 328.15426, 0.1%, retention time 13.5 min) and scoulerine (molecular mass m/z 328.15429, 0.02%, retention time 16.5 min), which were produced from THP by live mice and excreted in urine. Coreximine and scoulerine are typical and abundant tetrahydroprotoberberine alkaloids occurring in the plant kingdom.

To verify the formation of salutaridine with the correct stereochemistry, mice were injected (i.p.) with (*R*)-[*N*-CD$_3$]-reticuline (200 nmol per mouse) using the same injection protocol as for THP and the phenol-coupled products were isolated from the mouse urine and analyzed by HR-LC-MS. As shown in Fig. 3, the four phenol-coupled products [*N*-CD$_3$]-pallidine, [*N*-CD$_3$]-corytuberine, [*N*-CD$_3$]-salutaridine and [*N*-CD$_3$]-isoboldine were separated and exhibited identical retention times by HPLC on comparison with standard compounds. The *para-ortho* phenol-coupled biosynthetic product salutaridine had m/z 331.17326 (theory: m/z 331.17317) with a deviation from the theoretical value of 0.3 in parts per million (ppm) at a concentration of 52 pmol per mL mouse urine. The absolute configuration of the salutaridine formed has been previously established as (+) (24).

Transformation of Salutaridine to Thebaine. Live mice are capable of transforming THP and (*R*)-reticuline to salutaridine. Mammals, therefore, have a pathway leading from simple tetraoxygenated tetrahydrobenzylisoquinoline alkaloids to salutaridine. This morphinan intermediate is converted by poppy plants to thebaine (29, 30). The assumption can be made that there is a pathway in mammals, similar to that found in the poppy plant, that yields thebaine from salutaridine. Previous work from our laboratory (31) has shown that salutaridine is reduced stereoselectively at the expense of NADPH to salutaridinol but not to episalutaridinol. We synthesized salutaridinol chemically by reducing salutaridine in the presence of NaBD$_4$ and separated the resultant diastereomers by TLC. [7D]-Salutaridinol was injected (i.p.) into mice (200 nmol per mouse) to monitor a possible transition to thebaine. Urine was processed avoiding acid conditions and the recovered alkaloidal fraction was isolated and subjected to HR-LC-MS. The isolated alkaloid had a molecular mass of m/z 313.16556 and a composition of $C_{19}H_{21}{}^2HO_3N$, whereas unlabeled, standard thebaine had a mass of m/z 312.15934 and an elemental composition of $C_{19}H_{22}O_3N$. The HPLC retention time was 18.13 min for the labeled compound and 18.16 min for the unlabeled, standard thebaine. The fragmentation pattern

Table 1. Metabolites detected by HR-LC-MS in the urine of mice injected (i.p.) with (*R, S*)-THP

Alkaloid	Total number of alkaloids found	Amount (%)*	m/z (experimental)	Chemical formula
monomethoxy-tetrahydrobenzylisoquinoline	3	49	302.13857	$C_{17}H_{20}O_4N$
dimethoxy-tetrahydrobenzylisoquinoline	1	32	316.15424	$C_{18}H_{22}O_4N$
dimethoxy-*N*-methyl-tetrahydrobenzylisoquinoline	4	1.5	330.16997	$C_{19}H_{24}O_4N$
tetrahydroprotoberberine (tetrahydroxyberbine)	2	3	300.12286	$C_{17}H_{18}O_4N$
monomethoxy-tetrahydroprotoberberine	3	10	314.12286	$C_{18}H_{20}O_4N$
dimethoxy-tetrahydroprotoberberine	4	0.34	328.15426	$C_{19}H_{22}O_4N$
dimethoxy-*N*-methyl-aporphine	2	0.07	328.15429	$C_{19}H_{22}O_4N$
dimethoxy-*N*-methyl-morphinandienone	2	0.09	328.15431	$C_{19}H_{22}O_4N$

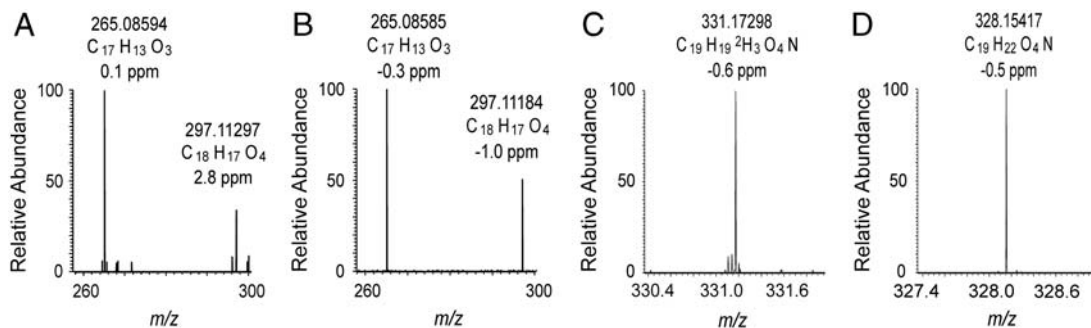*Percentage of the total amount of recovered alkaloids.

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Fig. 2. HR-LC-MS of salutaridine (m/z 328.15431) and [1, 3, 4-D_3]-salutaridine (m/z 331.17316) eluting at 15.4 min. (*A*) HR-LC-MS/MS of salutaridine/sinoacutine detected in urine of mice injected (i.p.) with (*R, S*)-THP. (*B*) HR-LC-MS/MS of salutaridine standard. (*C*) HR-LC-MS of [1, 3, 4-D_3]-salutaridine/sinoacutine detected in urine of mice injected (i.p.) with (*R, S*)-[1, 3, 4-D_3]-THP. (*D*) HR-LC-MS of salutaridine standard.

of labeled thebaine detected in urine of mice injected (i.p.) with [7D]-salutaridinol is shown in Fig. 4.

The labeled alkaloid is thebaine containing one deuterium atom presumably at position C7. The concentration of labeled thebaine was 128 pmol per mL mouse urine. The injection (i.p.) of the other diastereomer [7D]-episalutaridinol according to the same injection protocol did not result in thebaine formation. This demonstrates that the conversion of salutaridinol to thebaine is a stereoselective process when catalyzed in vivo. Chemical conversion of episalutaridinol to thebaine did not occur in the animal or during the processing of urine.

Formation of Morphine from Thebaine. Thebaine, the pentacyclic morphinan intermediate of the opium poppy plant, follows two biochemical routes to morphine. The *O*-demethylation of thebaine at the 6 position to yield neopinone and codeinone is followed by reduction to codeine, which in turn is *O*-demethylated at the 3 position to yield morphine. An alternative is the demethylation of thebaine initially in the 3 position to yield oripavine, with further 6-*O*-demethylation to morphinone, which is enzymatically reduced to morphine. It is remarkable that chemotypes of the poppy plant exist in which either route is dominant or even exclusive (32). We have demonstrated here that [7D]-thebaine is formed by mice after injection (i.p.) of [7D]-salutaridinol, as measured by excretion into urine. The question remains whether thebaine is further metabolized in the rodent and if so, whether the metabolic route leads to morphine. If morphine is formed from thebaine, is it formed by the pathways known in poppy, via codeine or via oripavine or both? The ability of humans to 3-*O*-demethylate codeine to morphine is established (33, 34). The enzyme responsible for this reaction was shown to be CYP 2D6 (35, 36). The activity of this P450 enzyme is under genetic control due to a polymorphism resulting in different phenotypes (37). Recently, human CYP 2D6 has been fully characterized with regard to morphine pathway substrates (*R*)-reticuline, thebaine and codeine and their respective products (24).
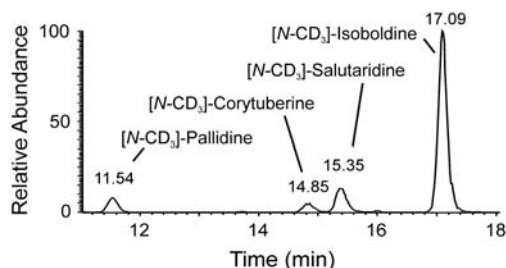
Synthetic [*N*-CD_3]-thebaine was injected (i.p.) (a total of 200 nmol per mouse) and three alkaloidal metabolites were found in the urine sample. Identification was achieved initially by comparison with the retention time of unlabeled potential morphinan alkaloids and subsequently confirmed by high resolution-mass spectrometry (HR-MS). The three metabolites were unequivocally identified as [*N*-CD_3]-codeine, [*N*-CD_3]-oripavine and [*N*-CD_3]-morphine (Fig. 5).

All three transformation products were verified by HR-MS with a high mass accuracy of <1 ppm. Quantitation of the three metabolites revealed that only a total of 5 pmol per mL urine was isolated from the urine of injected (i.p.) mice. Because acid hydrolysis is known to liberate the unbound morphinans from the known glucuronic acid or sulfate metabolites, the urine sample was hydrolyzed with HCl, however, the yield was not increased. Moreover, no residual [*N*-CD_3]-thebaine was present in the urine. Analysis of liver, brain, kidney, and blood did not reveal presence of any of the labeled morphinans. Excretion in the feces of these animals can be excluded because it is known that the feces contains $< 1 - 8\%$ of the total morphinans applied by injection (34, 38). The *N*-demethylated alkaloids northebaine, norcodeine, nororipavine, and normorphine were not detected in the mouse urine. The metabolic fate of the major portion of the injected thebaine remains unknown. It has been shown that codeine is 3-*O*-demethylated in vivo to morphine (33, 34). Oripavine was injected (i.p.) into mice to verify that morphine can also be produced in mammals via 6-*O*-demethylation of oripavine. Quantitative analysis revealed that only 0.3% of the total amount of injected (i.p.) substance was recovered. One of the urinary products was unequivocally verified after comparison of retention time and HR-MS with standard as morphine (−0.4 ppm) in a concentration of 2.1 pmol/mL urine.

Fig. 3. Full scan extracted chromatogram of metabolites with m/z 331.17326 detected in the urine of mice injected (i.p.) with (*R*)-[*N*-CD_3]-reticuline. The HR-LC-MS analysis revealed the urinary excretion of the stable-isotope labeled phenol-coupled products [*N*-CD_3]-pallidine, [*N*-CD_3]-corytuberine, [*N*-CD_3]-salutaridine and [*N*-CD_3]-isoboldine.
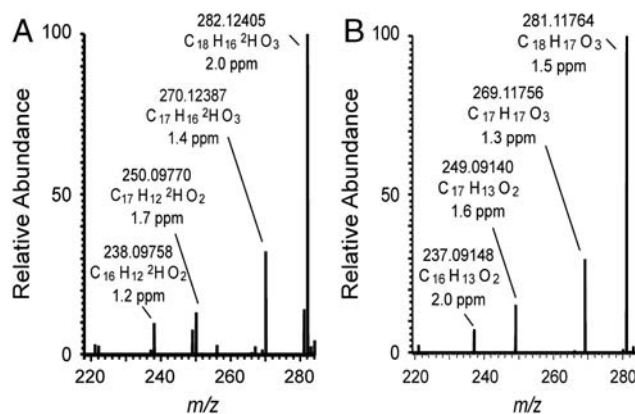

Fig. 4. HR-LC-MS/MS of [7D]-thebaine and thebaine standard eluting at 18.1 min. (*A*) HR-LC-MS/MS of [7D]-thebaine found in urine after injection (i.p.) of [7D]-salutaridinol into mice. (*B*) HR-LC-MS/MS of thebaine standard.
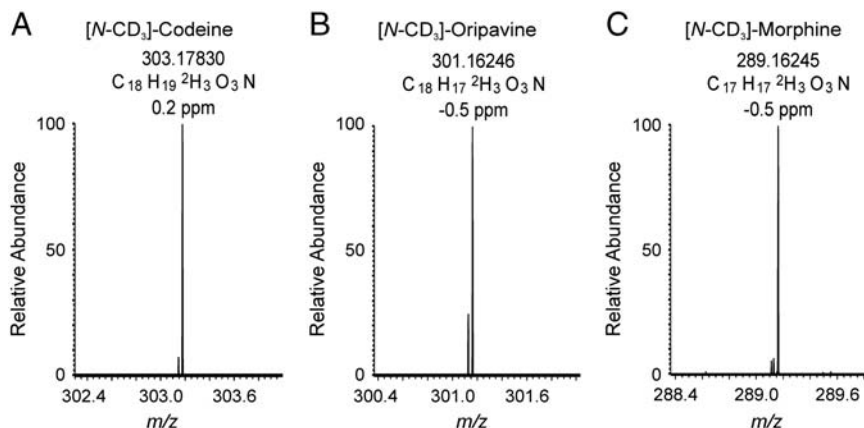
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Fig. 5. HR-LC-MS analysis of stable-isotope labeled metabolites detected in urine of mice injected (i.p.) with [N-CD$_3$]-thebaine. (*A*) HR-MS of [N-CD$_3$]-codeine (retention time 6.6 min). (*B*) HR-MS of [N-CD$_3$]-oripavine (retention time 10.2 min). (*C*) HR-MS of [N-CD$_3$]-morphine (retention time 2.9 min).

The biotransformation of oripavine to morphine as demonstrated here confirms that animals are capable of catalyzing the cleavage of the vinyl-ether in oripavine. Next to morphine, a second urinary metabolite with m/z 284.12803 (−0.2 ppm) that was found present after injection (i.p.) of oripavine was predicted to be morphinone. This assumption is supported by reports describing morphinone as an in vivo and in vitro metabolite of morphine in mammals (39, 40).

With the injection (i.p.) of proximal precursors of morphine into mice, it was verified for the first time that mammals are capable of synthesizing morphine from THP as depicted in Fig. 6. Surprisingly, the data suggest that a bifurcate pathway leads from thebaine to morphine via the intermediate codeine and simultaneously from oripavine, as in the opium poppy plant.

Discussion
Based on the findings that morphine is excreted in the urine of humans and rodents (7, 13–15), we studied the excretion of labeled morphine and its precursors after injection (i.p.) of potential alkaloidal precursors. This unique approach allows that the injected potential precursors are distributed in the blood, where they are metabolically modified, and then a small portion of these alkaloids are excreted in urine. These molecules thereby escape further modification and provide a static picture of the composition of the chemical constituents that have been formed in the organs of the animal. Using this approach and initially using the most distant potential alkaloidal precursor for mor-

phine, THP, we could demonstrate that salutaridine, an intermediate of the morphine pathway, was produced along with several other alkaloids that are already known from the plant kingdom. This is the first time that this phenol-coupled intermediate has been found in vivo in mammals. This is the second time that a phenol-coupling reaction has been found in the animal kingdom; the first example was the phenol-coupling involved in the formation of thyroxine in the thyroid gland (41). The morphinan intermediate formed from THP, salutaridine, through the phenol-coupling reaction of (R)-reticuline, is produced together with (−)-corytuberine, (+)-pallidine, and (−)-isoboldine. These four compounds were also characterized as products, derived via O- and N-methylation, in the urine of mice injected (i.p.) with (R,S)-THP. Injection (i.p.) of the naturally occurring reduction product of salutaridine, salutaridinol (29), into mice yielded thebaine in urine. This morphinan alkaloid is recognized as an intermediate (42) in the pathway to morphine. Indeed, injection of [N-CD$_3$]-thebaine into mice led to formation of [N-CD$_3$]-morphine and its two closest precursors, [N-CD$_3$]-codeine and [N-CD$_3$]-oripavine. Interestingly, the bifurcate pathway to morphine, which is known from opium poppy, is also realized in the mammalian morphine pathway. The early stages of the pathway in mammals, however, differ from that in the plant in that the biosynthetic route in mammals yields a tetrahydroxylated tetrahydrobenzylisoquinoline as the first alkaloidal intermediate, whereas that in the plant yields a trihydroxylated tetrahydrobenzylisoquinoline. This suggests that the
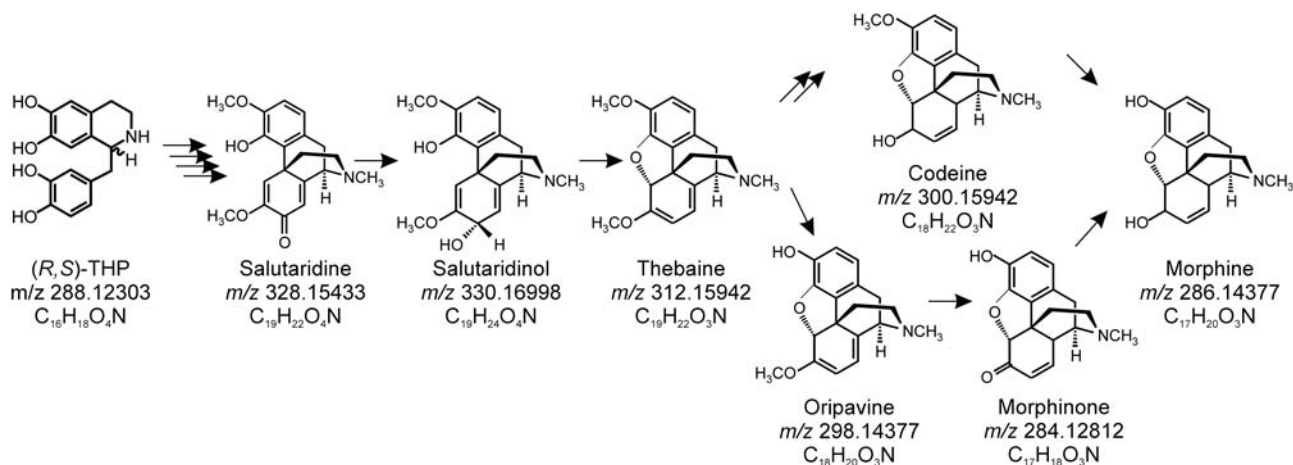

Fig. 6. Proposed biosynthetic pathway from THP to morphine in mammals. Morphine precursors and morphine were verified by HR-LC-MS as urinary metabolites of mice injected (i.p.) with biosynthetic precursors. The theoretical mass and chemical formula is shown for each detected metabolite.

Table 2. Summary of yields for intermediates and morphine excreted after injection (i.p.) of morphine precursors into mice

Injected (i.p.) alkaloid	Total amount of injected (i.p.) alkaloid/animal	Number of mice used	Urine, pooled (mL)	Detected alkaloid (%)[*]
(R, S)-THP	6.1 μmol	6	19.5	salutaridine/sinoacutine (0.003)
(R)-[N-CD$_3$]-reticuline	0.2 μmol	5	12.0	[N-CD$_3$]-salutaridine (0.062)
[7D]-salutaridinol	0.2 μmol	2	5.6	[7D]-thebaine (0.179)
[N-CD$_3$]-thebaine	0.2 μmol	8	19.7	[N-CD$_3$]-codeine (0.001); [N-CD$_3$]-oripavine (0.002); [N-CD$_3$]-morphine (0.002)
oripavine	0.2 μmol	2	6.8	morphine (0.004)

[*]In percentage of the dose administered.

morphine pathways in mammals and plants may have evolved independently.

An intriguing point is the low yield of intermediates and final products detected following injection (i.p.) of the alkaloidal precursors into mice (Table 2). Despite the sensitive analytical method used (Table 3), we were unable to detect morphine or labeled- and unlabeled precursors in either blood or in major organs. This will be addressed in future studies.

In contrast to other groups that reported on the detection of morphine in animal tissue (2, 7, 12), we never found morphine or precursors in properly worked-up animal organs such as brain, liver, kidney, and adrenal glands. Thorough analysis of the rodent chow (*Material and Methods*) did not reveal morphine or morphine precursors as well. In urine, the compounds applied, such as salutaridinol, thebaine, and oripavine, were only recovered in the range of about 0.1–1%; no trace amounts of these compounds, the corresponding *N*-demethylated alkaloids or their transformation products were found in mouse tissue. We assume that alkaloids were either strongly modified or degraded within the animal organs. However, 1% of the original injected alkaloids and their transformation products in urine were sufficient for HR-LC-MS analysis. The applied and transformed alkaloids detected in urine should be regarded as qualitative, not quantitative, measurements. Several of these morphinan biosynthetic precursors (oripavine and codeinone) and morphine itself will react with the opiate receptors and other binding components (43). Their biochemical fate after activation of the receptor is unknown. The concentration of morphine in human neuroblastoma cells detected by two independent groups (10, 12) and in mouse cerebellum (12) was found to be between 10–200 nM, a concentration that can stimulate the μ receptor in vivo (43). On account of our present finding that morphine may undergo rapid and extensive biochemical or compartmental inactivation, we assume that the de facto concentration of morphine within the microenvironment of the receptor may be considerably higher.

We have used urine analysis to demonstrate that live mammals contain the enzymatic machinery to synthesize morphine from the most distant alkaloidal precursor THP, the presence of which in human and rodent tissue had been amply shown. Important future goals will be an understanding of the nature of the enzymes that catalyze the transformation of THP to morphine and the identity of the underlying genes. This knowledge will then aid

in investigating the physiological role(s) of endogenous morphine in mammals.

Material and Methods

Animals. Four to eight month old female C57 black mice were purchased from Charles River. The animals were housed at the Animal Facility at Washington University. Free access to rodent chow (LabDiet 5001 Rodent Diet) and water was provided. Animal experiments were approved by the Animal Studies Committee at Washington University.

Injection into Mice and Collection of Urine. Substrate and reference compounds were obtained from our natural product collection. The isotopical distribution of stable-isotope labeled, injected (i.p.) precursors was as follows: for (R, S)-[1, 3, 4-D$_3$]-THP 3.1%[D$_2$]/85.5%[D$_3$]/11.4%[D$_4$], for (R)-[N-CD$_3$]-reticuline 0.1%[D$_0$]/0.2%[D$_1$]/0.5%[D$_2$]/99.0%[D$_3$]/0.3%[D$_4$], for [7D]-salutaridinol 9.6%[D$_0$]/77.8%[D$_1$]/12.5%[D$_2$], and for [N-CD$_3$]-thebaine 0.1%[D$_0$]/0.6%[D$_2$]/99.0%[D$_3$]/0.3%[D$_5$]. A sterile potential precursor solution containing 50–2000 nmol alkaloid per 200 μL water was injected (i.p.) into each mouse every 24 h for 4 d using a 1 mL hypodermic syringe with a 26-gauge needle (1/2 inch). This experimental setup was developed to collect enough urine for the HR-LC-MS analysis. Mice were housed during the 4 d of experiment in a metabolic cage, urine was collected every 24 h and stored at −20°C. During the experiment, free access was provided to water and rodent chow.

Isolation of Alkaloids. Tissue (0.4–1.5 g), pooled urine (1–5 mL), or blood (1 mL) obtained from mice was treated as described in (15) with slight modifications. After hydrolysis in 2 N HCl for 40 min at 110 °C, the sample was cooled and the pH was adjusted to 7–8 with 10 N KOH. The sample was loaded onto a Bond Elut Certify Solid Phase Extraction (SPE) cartridge (130 mg, 3 mL, Varian) that had been preconditioned with 2 mL methanol and 2 mL water. The cartridge was rinsed with 2 mL water, 1 mL acetate buffer pH 4.0, 2 × 2 ml methanol, and, after 2 min vacuum, eluted with 2 mL dichloromethane/isopropyl alcohol/ammonium hydroxide (8:2:0.2). The eluate was evaporated under a stream of nitrogen, resuspended in 100 μL water/methanol (8:2) containing 0.1% formic acid and subjected to HR-LC-MS analysis. Extraction of 100 g of rodent chow (LabDiet 5001 Rodent Diet) was

Table 3. Summary of limits of detection (LOD) for morphine and morphine precursors

Alkaloid	LOD urine (pmol/mL)	Standard deviation (±%)	LOD blood (pmol/mL)	Standard deviation (±%)	LOD tissue (pmol/g)	Standard deviation (±%)
THP	2	5	2	6	2	6
reticuline	1	3	1.5	6	1.4	4
salutaridine	3	4	3	5	3.3	8
salutaridinol	2	5	2.5	7	3	7.5
thebaine	1.7	4	2	5	2	6
oripavine	2	5	3	5.5	3	6
codeine	1.7	4	2	7	2	8
morphine	1.1	5	2	6.5	2	7

conducted in 2 N glacial acetic acid in methanol using a Soxhlet apparatus. The solvent was removed under vacuum and the reconstituted extract was subjected to SPE and analyzed by MS.

HR-LC-MS Analysis. Atmospheric pressure chemical ionization-fourier transform-mass spectrometry (APCI-FT-MS) spectra were obtained using an LTQ-Orbitrap Spectrometer (Thermo Fisher). The spectrometer was operated in positive mode (1 spectrum s-1; mass range: 50–1,000) with a nominal mass resolving power of 60,000 at m/z 400 at a scan rate of 1 Hz using automatic gain control to provide high-accuracy mass measurements (≤2 ppm deviation). The internal calibration standard bis-(2-ethylhexyl)-phthalate (m/z 391.28428) was used for the determination of elemental composition. The spectrometer was equipped with a Surveyor HPLC system (Thermo Scientific) consisting of LC-Pump, UV detector ($\lambda = 254$ nm), and autosampler (injection volume 10 μL). Separation of samples was achieved by using a Synergi Fusion RP HPLC column (Phenomenex, 4 μm, 150×3 mm) combined with a Synergi Fusion RP guard column

(Phenomenex, 4×3 mm). The mobile phase total flow was set to 0.5 mL/min with binary gradient elution, using solvents A (0.1% formic acid, 10 mM ammonium acetate) and B (0.1% formic acid in acetonitrile) (all v/v). The gradient started with 5% B for 4 min and was increased to 30% B over 20 min. Elution was continued for 10 min at 100% B followed by a 7 min equilibration with the starting condition. MS/MS spectra were obtained from the corresponding parent ions ($[M + H]^+$) with a collision energy of 35 V for salutaridine and 40 V for thebaine. For quantification all external calibration graphs were linear ($R^2 > 0.99$) in the range of 0.35–35 pmol.

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