Transformation of Morphine by Resting Cells and Cell-Free Systems of *Arthrobacter* Sp.

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Morphine can be transformed into 14-hydroxymorphine and a related unidentified material by resting cells of an *Arthrobacter* species. Cell-free extracts containing the transforming enzyme(s) have been obtained. O_2, Fe^{2+}, and reduced nicotinamide adenine dinucleotide stimulate the transformation.

Transformation of steroids by microorganisms has been a widely used procedure to produce commercial preparations of physiologically active steroids (4). Other complex natural products have also been examined as substrates in microbial transformation, for example, ergot (2), indoles (5), yohimbines (6), or opium alkaloids (8). Cell-free systems have been described which transform nicotine (7) and steroids (14, 15), and the hydroxylation of the main constituents of marijuana by rat liver extracts has been well studied (3). We have previously reported the hydroxylation of morphine and codeine by cultures of an *Arthrobacter* species (P. Liras et al., *In* L. A. Unterkofler (ed.), *Developments in Industrial Microbiology*, vol. 16, in press). Such transformation of morphine might result in new analgesic, possibly nonaddictive derivatives, and transforming enzymes are promising tools as reagents to detect trace amounts of morphine. The present work describes the favorable conditions for hydroxylation of morphine by cultures and cell-free extracts of this strain, and a partial purification of the involved enzyme has been achieved.

MATERIALS AND METHODS

Growth conditions. Cells were grown in 1-liter flasks containing 300 ml of 0.5% K_2HPO_4, 0.5% yeast extract, and 0.5% tryptone. Three milliliters of a 24-h culture were used as inoculum. The cells were grown at room temperature on a 2-in. (5.08-cm) throw rotary shaker (150 rpm) for 20 h, centrifuged at 5,000 × g for 10 min, washed twice with 10 mM phosphate buffer, pH 7.5, and suspended in the same buffer at a concentration of 10 mg of dry weight/ml. Cell suspensions were stored frozen at −20 C without appreciable loss of activity for at least 3 months.

Preparation of cell-free extracts. The cells were suspended at 25 mg of dry weight/ml of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (20 mM, pH 8.4) and were sonically treated for 2 min,

in four intervals of 30 s each, in a sonifier cell disruptor, model W 185D (Heat Systems Ultrasonic, Inc., Plainview, Long Island, N.Y.). The cells were kept at 4 C. The extracts were centrifuged twice at 5,000 × g for 10 min, and the pellets were discarded. The yellow supernatant was centrifuged at 18,000 × g for 40 min in a Sorvall RC-2 centrifuge, producing a gelatinous pellet (P_1). The remaining supernatant was centrifuged at 102,000 × g for 60 min in a Spinco Model L centrifuge, producing a pellet, P_2, and the final supernatant. Both pellets were suspended separately in the original volume of 20 mM Tris buffer, pH 8.4, containing 0.03% Triton X-100 and sonically treated for 10 s to produce a finely divided, almost transparent suspension. The processes were carried out at 4 C, and the cell-free preparation was assayed immediately.

Assay of hydroxylating activity. Whole cells were suspended at 4 mg of dry weight/ml in 10 mM phosphate buffer, pH 7.5, containing 100 µg of morphine/ml, 20 mmol of $FeSO_4$, and 0.05 µCi [*N-methyl*-^{14}C]morphine (500 µg of morphine/ml), in a total volume of 4 ml. The cell suspension was incubated with shaking at 30 C.

In cell-free extracts, the reaction was started by adding the desired amount of enzymes to the 25-ml flasks containing 60 mmol of Tris-hydrochloride buffer, pH 8.4, 0.05 µCi [*N-methyl*-^{14}C]morphine, 500 µg of morphine/ml, 15 mmol of $FeSO_4$, and 4.8 mmol of reduced nicotinamide adenine dinucleotide (NADH) in a final volume of 3 ml. The flasks were incubated at 30 C with shaking up to 18 h, and the reaction was stopped by addition of 10% NH_4OH (vol/vol). To avoid contamination, the system was occasionally sterilized through membrane filters (Millipore Corp.) before incubation, although no difference in the results was found.

Extraction and determination of transformed products. After incubation the cultures or extracts were adjusted to pH 10 with ammonium hydroxide and extracted three times with 2 volumes of chloroform:ethanol (2:1 vol/vol) for 40 min each. The mixed extracts were dried in an air stream, and the residue was suspended in methanol and spotted on silica gel G plates (20 by 20 cm), 250 microns (Analtech, Inc.). The

plates were developed using the solvent system ethyl acetate:methanol:ammonium hydroxide (14.8 M) (86:10:4) and were visualized with I_2 vapors. Standard samples of 14-hydroxymorphine were always included on the plates. The plates were scraped off, the silica gel was placed into radioactivity vials, and the I_2 was allowed to evaporate before the scintillation cocktail was added. Toluene containing 4 g of 98% 2,5-diphenyloxazole/2% Bis MSB per liter (Omni-scint, ICN, Inc.) was used as scintillation cocktail. Radioactivity was measured in a Beckman LS-230 model liquid scintillation counter.

Protein. Protein was determined spectrophotometrically, measuring optical density at 260 and 280 nm (10).

Chemicals. [*N-methyl-*^{14}C]morphine hydrochloride (143 Ci/mg) was purchased from Amersham/Searle (Arlington Heights, Ill.). Morphine and codeine were obtained from Merck and Co. (Rahway, N.J.). 14-Hydroxymorphine was a kind gift from Everette L. May of the National Institutes of Health (Bethesda, Md.).

RESULTS

Transformation products: factors affecting the kinetics of transformation. Two main transformation products are formed when cells or cell-free extracts of *Arthrobacter* sp. 86 are incubated with morphine. Thin-layer and gas chromatography parameters of those substances have already been described and one of the products has been identified as 14-hydroxymorphine (P. Liras et al., *In* L. A. Unterkofler (ed.), in press). The structure of the second substance formed, product "C" has not yet been established. Both 14-hydroxymorphine and transformation product C are the main components formed from morphine by cells, and are formed by whole cells in about the same proportion under the different conditions described below. Minor proportions of other unknown products were detected, but they are not considered in this study. A nonspecific degradation of morphine also occurs, as observed by the quantitative difference between the total initial morphine, the morphine transformed, and the morphine remaining in the medium. This material appears as radioactivity in the aqueous phase after extraction, but thin-layer chromatography and gas chromatography showed that this radioactivity is not present as a single component.

The maximum yield of both transformation products is reached after 3 days of incubation of morphine with a cell suspension of 4 mg of dry weight/ml (Fig. 1). The time required for maximum production of both transformation products was inversely proportional to the concentration of cells in the preparation. The trans-
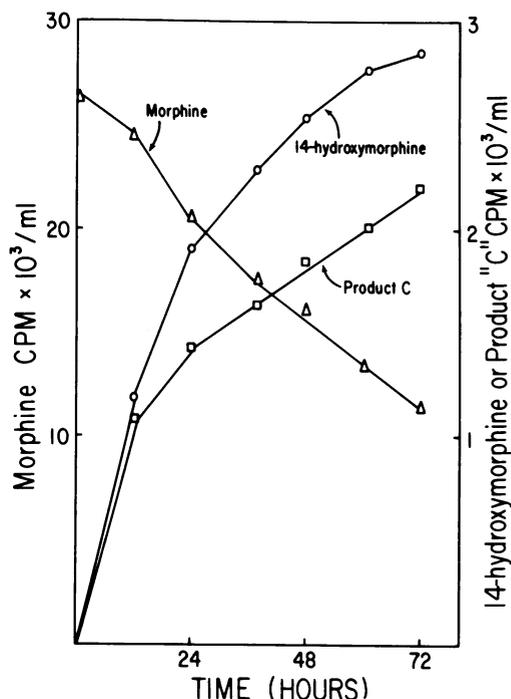

FIG. 1. *Transformation of morphine and formation of products. Cells (4 mg/ml) were incubated in the presence of 100 µg of morphine per ml (0.05 µCi), 20 mmol of FeSO₄, and 40 mmol of buffer, pH 7.5. The total volume was 4 ml.*

forming process was greatly facilitated by increasing the dissolved oxygen in the cell preparation (Fig. 2). An increase in aeration from about 150 to 500 ml of O_2/h produced a substantial increase in 14-hydroxymorphine production, although no net increase in the cell mass occurred. Higher aeration—up to 6,000 ml of O_2/h—further increased the production but not proportionally to the increase in aeration. Cells under anaerobiosis did not transform or degrade morphine. Although partial cell death takes place under anaerobiosis, this is not the only explanation for the lack of transformation, since cell-free extracts of *Arthrobacter* sp. do not degrade morphine under anaerobiosis (Table 1).

The transformation does not appear to require the induction of specific enzymes. Cells which had been preincubated for 12 h with or without morphine, when transferred to fresh medium plus morphine, produced approximately the same yield of products at the same transformation rate. Young cells transformed morphine at a faster rate than older cells.

The effect of some reagents on the hydroxylating activity of the cells was studied.
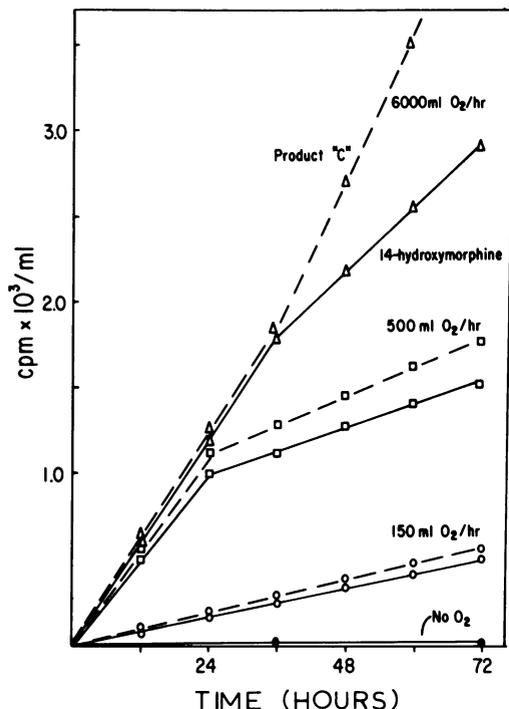
FIG. 2. *Influence of aeration on morphine transformation. Cells (4 mg/ml) were incubated as indicated in Materials and Methods, under the different aeration conditions indicated. Flasks of 125 ml containing 20 ml of media were used: (□) no shaking, (○) 150 rpm, (△) 150 rpm, saturated by bubbling air, and (●) 150 rpm, under argon atmosphere. The aeration was calculated according to Smith and Johnson (13).*

Ethylenediaminetetraacetic acid at concentrations above 1 mM partially or completely inhibited the formation of 14-hydroxymorphine, 5 mM being completely inhibitory (Table 2). This suggested that metal cations may be involved in the hydroxylation. Consequently, the effect of different cations was tested. At 2 mM concentration, Zn^{2+} (data not shown) and Cu^{2+} almost completely inhibited the transformation of 14-hydroxymorphine and product C; Mo^{2+}, Mn^{2+} and Mg^{2+} had no effect (data not shown), and Fe^{2+} clearly increased the yield of products. Different iron concentrations produced an increase in the formation of 14-hydroxymorphine with a maximum effect at 5 mM, higher concentrations being inhibitory (Table 2). The formation of product C is not affected or is slightly inhibited by the presence of iron. Fe^{3+} produced a similar effect as Fe^{2+} although possibly both cations reach an equilibrium in the incubation media. In the presence of iron, the decrease of morphine in the medium was

TABLE 1. *Effect of pH, Fe^{2+} ions, oxygen, and reduced cofactors on morphine transformation by cell-free extracts of Arthrobacter sp. 86[a]*

Expt no.	Conditions	14-Hydroxymorphine (counts/min per ml)	Product C (counts/min per ml)
1	Cell-free extract, pH 8.4, 15 mmol of Fe^{2+}, 4.8 mmol of NADH	5,940	5,360
2	Cell-free extract, pH 8.4, 15 mmol of Fe^{2+}, 4.8 mmol of NADH	4,260	3,960
3	Cell-free extract, pH 8.4, 15 mmol of Fe^{2+}	3,380	4,340
4	Cell-free extract, pH 7.0, 15 mmol of Fe^{2+}	169	217
5	Cell-free extract, pH 8.4	2,010	2,029
6	As expt no. 1, but under argon atmosphere	260	300
7	60 mmol of Tris-hydrochloride buffer, pH 8.4, 15 mmol of Fe^{2+}, 4.8 mmol of NADH	200	150

[a] Five hundred milligrams (dry weight) of cells were broken, centrifuged at 10,000 × g, and the cell-free supernatant was assayed for hydroxylating activity under the conditions indicated below. The reaction mixture contained 500 μg morphine per ml (33.000 counts/min per ml), the additions indicated above, and either 2.6 ml of cell-free extract adjusted to the indicated pH, or 2.6 ml of Tris-hydrochloride buffer, 20 mM, pH 8.4, in a total volume of 3 ml. The system was incubated for 15 h at 30 C.

smaller than in its absence (Table 2), therefore producing a much higher specific transformation of the drug. This fact was also found in cell-free preparations. Glutathione at a concentration of 5 mM does not affect the formation of 14-hydroxymorphine, but increases the yield of product C at least three times.

Hydroxylation of morphine by cell-free extracts of Arthrobacter sp. 86. Cell-free extracts of *Arthrobacter* sp. hydroxylated morphine at about the same rate than whole cells.

Table 1 shows the effect of pH, iron, oxygen, and reduced cofactors on the hydroxylation of morphine. Both NADH and reduced nicotinamide adenine dinucleotide phosphate (NADPH) produced an increase in the hydrox-

TABLE 2. *Effect of EDTA[a], glutathione, Cu^{2+}, and Fe^{2+} ions on the transformation of morphine by resting cells of Arthrobacter sp. 86[b]*

Additions (mM)	Morphine in the medium at 72 h (counts/min per ml)	14-Hydroxymorphine (counts/min per ml)	Product C (counts/min per ml)
None	16,500	5,600	5,450
Glutathione (5)	15,228	5,220	13,400
EDTA (4)	29,330	424	632
CuSO₄ (2)	4,608	560	320
FeSO₄ (1)	19,820	8,200	4,400
FeSO₄ (2)	20,860	9,130	4,450
FeSO₄ (5)	21,200	10,454	4,360
FeSO₄ (10)	27,113	6.680	4,120

[a] EDTA, Ethylenediaminetetraacetic acid.

[b] Cells (4 mg/ml) in 10 mM phosphate buffer, pH 7.5, with 100 µg of morphine per ml (0.02 µCi/ml; 40,250 counts/min per ml), and the indicated additions were incubated for 72 h at 30 C.

TABLE 3. *Morphine-transforming activity in differential centrifugation fractions of Arthrobacter cell-free system[a]*

Fraction	14-Hydroxymorphine (counts/min per ml)	Product C (counts/min per ml)	Protein (mg/ml)
100,000 × g supernatant	6,425	2,055	11.6
20,000 × g pellet (P₁)	260	160	10.5
100,000 × g pellet (P₂)	250	210	3.2
Supernatant + P₁ + P₂	5,935	2,324	22.3
Total cell-free extract	4,718	1,987	16.6

[a] Cell-free extracts were prepared and hydroxylating activity was assayed as indicated in Materials and Methods. The reaction mixture contained at time zero 500 µg morphine per ml (33,100 counts/min per ml) and was incubated for 16 h at 30 C.

TABLE 4. *Hydroxylating activity of ammonium sulfate-fractionated extract of Arthrobacter sp. 86[a]*

Fraction	14-Hydroxymorphine (counts/min per ml)	Product C (counts/min per ml)	Protein (mg/ml)
Total cell-free extract (100,000 × g supernatant)	4,747	802	18.2
0 to 20% ammonium sulfate saturation	159	75	29.5
20 to 35% ammonium sulfate saturation	263	172	11.7
35 to 60% ammonium sulfate saturation	7,403	2,572	25.0
60 to 100% ammonium sulfate saturation	267	81	7.2
Ammonium sulfate-soluble fraction	198	54	3.9
Control	41	35	

[a] Cell-free extracts were prepared as in Materials and Methods, and the 100,000 × g supernatant was fractionated with solid ammonium sulfate. The fraction precipitated was collected by centrifugation at 5,000 × g during 10 min, and redissolved in 5 ml of phosphate buffer, 50 mM, pH 8, and then dialyzed overnight at 4 C against 25 mM phosphate buffer, pH 7.5. The reaction mixture contained 500 µg of morphine per ml (37,000 counts/min per ml), 2.8 ml of cell-free extract adjusted to pH 8.4, 4.8 mmol of NADH, and 15 mmol of Fe^{2+} in a total volume of 3 ml. The system was incubated for 16 h at 30 C.

ylation of morphine, although NADH was more effective. The specific decrease in absortion of NADH at 340 nm could not be detected in the presence of morphine, probably because several other enzymes in the cell-free preparation also oxidized the cofactor. The oxidized cofactors NAD^+ and $NADP^+$ did not affect the transforming activity in the cell-free extracts. Iron stimulated the hydroxylating activity of cell-free extracts as well as of whole cells.

The pH of the medium affected differentially the yield of 14-hydroxymorphine and product C by whole cells, resulting in a twofold increase of 14-hydroxymorphine when the pH was raised from 7 to 9 (data not shown; T. Atherholt, personal communication). Transformation by cell-free systems was even more affected by the pH than the transformation by the whole cells, in the previously indicated range of pH (Table 1, experiments no. 3 and 4).

A considerable difference in the amount of 14-hydroxymorphine and product C formed appeared in different cell-free preparations (Tables 3 and 4), although both were produced in the same proportion by treatment of morphine by whole cells (Fig. 1, Table 2).

The hydroxylating activity was completely recovered in the soluble fraction after centrifugation at 100,000 × g (Table 3). Addition of one or both pellets did not increase the activity. All the enzymatic activity precipitated between 35 and 60% saturation of ammonium sulfate. The activity for formation of 14-hydroxymorphine and product C appeared together in the same fraction (Table 4). However, the relative pro-

portion of both substances differs widely from one enzyme preparation to another. Conditions

which positively affect the formation of 14-hydroxymorphine may also affect positively the formation of product C (as NADH and NADPH, or pH) or have a negative effect (as Fe^{2+} ions). These results, together with the fact that product C formation is highly variable, indicate two separate enzymatic activities.

DISCUSSION

Transformation of morphine by resting cells, cell-free extracts and ammonium sulfate fractionated extracts of *Arthrobacter* sp. 86 was studied. 14-Hydroxymorphine was identified as one of the transformation products (P. Liras et al., *In* L. A. Unterkofler (ed.), in press).

The formation of 14-hydroxymorphine is a process requiring oxygen (Fig. 2; Table 1, experiment no. 6), which suggests that the oxygen of the hydroxyl group in 14-hydroxymorphine comes from the air, as has been shown in the formation of 14-hydroxycodeinone from thebaine (1). The enzymatic hydroxylating activity is partially inhibited by ethylenediamine tetraacetic acid, Zn^{2+}, and Cu^{2+} and stimulated by Fe^{2+}, NADH, and NADPH (Table 1). Hydroxylation partially takes place in the absence of external NADH, presumably due to endogenous coenzyme present in the cell-free extracts.

Other biological systems involved in drug transformation, such as the mono-oxygenase system present in rat liver microsomes (3), also requires NADPH, NADH, and atmospheric oxygen. In microorganisms, mixed function oxygenases constitute a coupled complex of soluble enzymes (9, 12). In *Pseudomonas* sp., this system is formed by at least three protein components (9, 11, 12) and requires Fe^{2+}, NADH, and molecular oxygen. The similarity in requirements for maximal activity in the morphine-transforming system suggests that enzymes similar to those mentioned above may be responsible for the transformation of the drug, although in our case they appear to be non-morphine-inducible enzymes.

It seems that the formation of product C is not catalyzed by the same enzyme or enzyme complex as the 14-hydroxylation of morphine, since the response of each activity to iron is different from the other. This is in agreement with the report that substance C appears as a product of transformation of 14-hydroxymorphine itself (P. Liras et al., *In* L. A. Unterkofler

(ed.), in press). However, adequate amounts of pure, radioactive 14-hydroxymorphine are not yet available to make a more complete study of this transformation.

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