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GUINEA-PIG LIVER MORPHINE 6-DEHYDROGENASE AS A NALOXONE REDUCTASE

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Abstract—Elution profiles of guinea-pig liver naloxone reductase and morphine 6-dehydrogenase on Mātrex green A, Sephadex G-100 and DEAE-cellulose (DE32) column chromatography used sequentially in the purification procedure were identical. The ratios of the two enzyme activities were almost constant throughout all the purification steps. The two enzymes were similarly more stable at pH 6.0 than at pH 8.0 on storage at 4°. The reversible inactivation of the two enzymes by the removal of 2-mercaptoethanol from the enzyme solution was the same. Inhibitory effects of lithocholic acid, $CuSO_4$, quercitrin, phenylarsine oxide, and prostaglandin E_1 on the two enzymes were almost the same. These results indicated that naloxone reductase is identical to morphine 6-dehydrogenase in the guinea-pig liver. For the reduction of naloxone, the enzyme utilized either NADPH or NADH as cofactor, and pH optima were 6.8 with NADPH and 6.2 with NADH. The K_m values for NADPH and NADH were 6.5 and 2.2 μM respectively. The V_{max} values for naloxone were 1.2 units/mg protein with NADPH and 0.5 unit/mg protein with NADH. The K_m values for naloxone were 0.27 mM with NADPH and 0.44 mM with NADH. The reaction product formed by the enzyme was identified as 6α-naloxol by thin-layer and gas–liquid chromatographic analyses. Accordingly, it is clear that the enzyme catalyzes the stereospecific reduction of naloxone to form the 6α-hydroxyl congener.

Naloxone is therapeutically useful for its ability to reverse opiate-induced respiratory depression, and its metabolism has been studied extensively in humans and various animal species. Naloxone is metabolized by glucuronide conjugation at the C-3 hydroxyl group and/or reduction of the C-6 oxo group to the corresponding 6α- and/or 6β-naloxol [1–8]. The liver cytosols of pig, cow, horse, guinea pig, rat, rabbit, chicken and turkey were capable of reducing naloxone to 6α- and/or 6β-naloxol in the presence of NADPH and to a lesser extent with NADH [9–13]. In addition, the presence of the reducing activity responsible for conversion of naloxone to 6α- and/or 6β-naloxol has been demonstrated in the liver cytosols of many vertebrate species other than the above mammalian and avian species [14]. Naloxone reductases of chicken, rabbit and guinea pig were partially characterized using the enzyme fractions obtained by ammonium sulfate precipitation of liver cytosols [9–11], and rabbit liver enzymes have been resolved into several species by isoelectric focusing [12, 13]. No further purification and characterization of naloxone reductase have been reported.

Recently, we purified and characterized morphine 6-dehydrogenase, which catalyzes the dehydrogenation of the C-6 hydroxyl group of morphine to morphinone, from guinea-pig liver cytosol [15]. Subsequently, it was found that the enzyme also participates in the reverse reaction (reduction of the C-6 oxo group of morphinone to morphine) and is competitively inhibited by naloxone. From these findings, we presumed that the reduction of naloxone in the guinea-pig liver is catalyzed, at least in part, by morphine 6-dehydrogenase.

To elucidate the relation between morphine 6-dehydrogenase and naloxone reductase in guinea-pig liver, we examined the elution profiles of the two enzymes at each step in the column chromatographic sequence utilized for the purification of morphine 6-dehydrogenase and the ratios of the two enzyme activities at each purification step. Several properties of the two enzymes were also compared. The results presented in this paper constitute evidence that naloxone reductase is identical to morphine 6-dehydrogenase. Stereospecific reduction of naloxone to 6α-naloxol by the enzyme is also demonstrated.

MATERIALS AND METHODS

Materials. Mātrex green A was obtained from the Amicon Corp. (Lexington, MA, U.S.A.); Sephadex G-100 and G-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden); DEAE-cellulose (DE32) was from Whatman Biochemicals Ltd. (Maidstone, Kent, U.K.); Ampholine carrier ampholyte (pH range 6–8) was from LKB Produkter AB (Bromma, Sweden); and precoated silica gel plate G60 (F254) was from E. Merck AG (Darmstadt, F.R.G.). Pyridine nucleotides were purchased from the Oriental Yeast Co., Ltd. (Osaka, Japan); and morphine hydrochloride was from Takeda Chemical Industries (Osaka, Japan). Naloxone hydrochloride was donated by the Sankyo Co., Ltd. (Tokyo, Japan). 6α- and 6β-Naloxol were prepared by reduction of naloxone with sodium borohydride in absolute ethanol and with formamidinesulfinic acid as described by

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Chatterjie *et al.* [16] respectively. All other reagents were of the highest grade available.

Enzyme purification. All the procedures for the purification of the enzyme were carried out as described previously [15]. Briefly, the enzyme was purified from the 105,000 *g* supernatant fraction of the guinea-pig liver by ammonium sulfate precipitation followed by Mātrex green A column chromatography, Sephadex G-100 gel filtration, and DE32 column chromatography.

Enzyme assay and protein determination. The activities of naloxone reductase and morphine 6-dehydrogenase were measured by the change in absorbance at 340 nm employing a 1 cm light path in a Shimadzu UV 150 spectrophotometer at 25°. The standard incubation mixture for naloxone reductase activity contained 1 mM naloxone, 0.17 mM NAD(P)H, enzyme solution and buffer in a total volume of 1.5 ml. Buffers used were 67 mM sodium phosphate buffer, pH 6.8, with NADPH and, pH 6.2, with NADH. For the assay of morphine 6-dehydrogenase, the standard incubation mixture contained 1 mM morphine, 1 mM NADP, enzyme solution and 100 mM Tricine–NaOH buffer, pH 9.1, in a total volume of 1.5 ml. One unit was defined as the amount of enzyme which catalyzes oxidation or reduction of 1 μmole of cofactor per min at 25°. Protein concentration was determined by the method of Lowry *et al.* [17] after all the protein was precipitated according to the method of Folin and Wu [18]. These methods were standardized with bovine serum albumin.

Isoelectric focusing. Isoelectric focusing was carried out at 4° with the aid of an LKB 8101 electrofocusing column containing a pH gradient of 6–8 in a 0–50% sucrose gradient. After electrofocusing for 48 hr under a constant voltage of 600 V, fractions of 2 ml were collected and monitored for enzyme activities, absorbance at 280 nm, and pH.

Identification of reduction product of naloxone by enzyme. A reaction mixture containing naloxone (6 μmoles), NADPH (6 μmoles), purified enzyme (20 milliunits) and 67 mM sodium phosphate buffer, pH 6.8, in a total volume of 6 ml was incubated for 30 min at 37°. After incubation, the reaction mixture was poured directly onto a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, U.S.A.), washed with 10 ml of water, and then eluted with 3 ml of methanol. The methanol eluate was analyzed for identification of the reaction product by thin-layer chromatography (TLC) using $CHCl_3$/methanol/28% NH_4OH (90:10:4, by vol.) as a solvent system and gas–liquid chromatography (GLC). In GLC analysis, the instrument employed was a Shimadzu gas chromatograph GC-3AM with a flame ionization detector. A glass column (3 mm × 3 m) packed with 3% OV-225 on Gas-Chrome Q (100–120 mesh) was used. Flow rates of nitrogen, hydrogen and air were 40, 30 and 950 ml/min respectively. The column temperature was maintained isothermally at 245°.

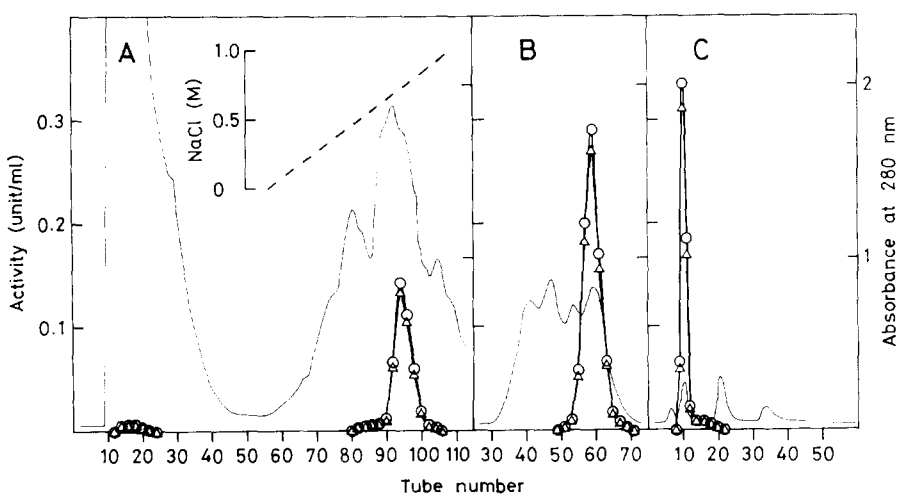

Fig. 1. Elution profiles of naloxone reductase and morphine 6-dehydrogenase on a Mātrex green A column (A), a Sephadex G-100 column (B), and a DE32 column (C). Naloxone reductase activity and morphine 6-dehydrogenase activity were measured with NADPH and NADP, respectively, as described under Materials and Methods. Key: (—○—) naloxone reductase activity; (—△—) morphine 6-dehydrogenase activity; (——) absorbance at 280 nm; and (– – –) concentration of NaCl. (A) Mātrex green A column chromatography. The ammonium sulfate fraction dialyzed against 50 mM sodium phosphate buffer, pH 8.0, containing 100 mM 2-mercaptoethanol (buffer A) was applied to a column of Mātrex green A (2.6 × 26 cm). The column was washed with buffer A until the absorbance of the eluate at 280 nm decreased to less than 0.1, and then eluted with a linear gradient formed by adding 250 ml each of buffer A and buffer A containing 1 M NaCl. Fractions of 10 ml were collected at a flow rate of 15 ml/hr. (B) Sephadex G-100 column chromatography. Mātrex green A fraction (tubes 91–100) was concentrated and applied to a column of Sephadex G-100 (2.6 × 90 cm), and then eluted with buffer A. Fractions of 5 ml were collected at a flow rate of 15 ml/hr. (C) DE32 column chromatography. Sephadex G-100 fraction (tubes 55–64) was concentrated and then dialyzed against 5 mM sodium phosphate buffer, pH 8.0, containing 100 mM 2-mercaptoethanol (buffer B). The dialysate was applied to a column of DE32 (1.6 × 90 cm) and eluted with a linear gradient formed by adding 300 ml each of buffer B and buffer A. Fractions of 10 ml were collected at a flow rate of 10 ml/hr.

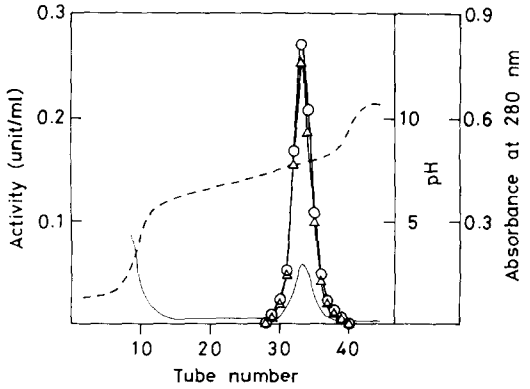

Fig. 2. Isoelectric focusing of DE32 fraction. A portion of DE32 fraction (tubes 9–11) was focused in a pH 6 to 8 gradient as described in Materials and Methods. Key: (—○—) naloxone reductase activity; (—△—) morphine 6-dehydrogenase activity; (——) absorbance at 280 nm; and (---) pH.

and the detector and injector temperatures were 270°.

RESULTS

Relation between naloxone reductase and morphine 6-dehydrogenase during purification. Although a very weak naloxone reductase activity appeared in an unadsorbed protein fraction, the remainder was eluted at around 0.75 M NaCl as a single peak of enzyme activity on Mātrex green A column chromatography (Fig. 1A). The elution pattern of the naloxone reductase activity was consistent with that of the morphine 6-dehydrogenase activity. The active fraction (tubes 91–100) was then applied to a column of Sephadex G-100. As shown in Fig. 1B, the naloxone reductase activity and the morphine 6-dehydrogenase activity were concurrently eluted as a single peak. The active fraction (tubes 55–64) was further applied to a column of DE32. Elution patterns of the two enzyme activities were identical and the peak of the enzyme activity was in good agreement with the second peak of protein (Fig. 1C). The active fraction (tubes 9–11) exhibited electrophoretically homogeneous protein [15]. Figure 2 shows the result of sucrose density gradient electrofocusing of the DE32 fraction. The peak of the naloxone reductase activity appeared at pH 7.6 and coincided with that of the morphine 6-dehydrogenase.

The two enzyme activities were similarly recovered throughout all the purification steps, and the enzyme fraction obtained at the final step showed 30% of the naloxone reductase activity and 32% of the morphine 6-dehydrogenase activity found in the soluble fraction (Table 1). The ratios of the two enzyme activities were almost constant throughout all the purification steps, and a very good approximation was observed at the steps after Mātrex green A column chromatography.

Stability. Morphine 6-dehydrogenase was more stable at pH 6.0 than at pH 8.0 on storage at 4°, and its activity was rapidly lost by the removal of 2-mercaptoethanol from the enzyme solution [15]. Similarly, the enzyme stored for a week in 50 mM sodium phosphate buffer, pH 8.0, containing 100 mM 2-mercaptoethanol lost more than 50% of the naloxone reductase activity, whereas more than 80% of the activity was still retained after a week in the case where the enzyme was stored in 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM 2-mercaptoethanol. When 2-mercaptoethanol was removed from the enzyme solution by gel filtration on Sephadex G-25 against 50 mM sodium phosphate buffer, pH 8.0, the naloxone reductase activity disappeared within 24 hr; however, the lost activity was recovered by the addition of 2-mercaptoethanol to the enzyme solution or the incubation mixture. These effects were the same as for morphine 6-dehydrogenase.

Effect of inhibitor. Table 2 shows the effects of various compounds on naloxone reductase and morphine 6-dehydrogenase. The two enzymes were strongly inhibited by lithocholic acid and $CuSO_4$ at 0.1 mM. Quercitrin, phenylarsine oxide and prostaglandin E_1 were also inhibitory. These compounds had a similar inhibitory effect on the two enzymes. Inhibition by ketamine was stronger toward morphine 6-dehydrogenase than toward naloxone reductase. However, when the buffers used in the assay of naloxone reductase and morphine 6-dehydrogenase were the same, the two enzymes were similarly inhibited by ketamine. For example, 31%

Table 1. Total activities and specific activities of naloxone reductase and morphine 6-dehydrogenase, and the ratios of the two enzyme activities in each purification step*

	Naloxone reductase			Morphine 6-dehydrogenase			
Fraction	Total activity (units)	Specific activity (milliunits/mg)	Yield (%)	Total activity (units)	Specific activity (milliunits/mg)	Yield (%)	Ratio
Soluble†	20.7	4.4	100	18.2	3.9	100	1.13
45–80% $(NH_4)_2SO_4$	17.5	7.7	84	14.8	6.5	81	1.18
Mātrex green A	10.4	152.9	50	9.9	145.2	54	1.05
Sephadex G-100	8.2	574.2	40	7.8	544.4	43	1.05
DE32	6.1	896.6	30	5.8	855.5	32	1.05

* Naloxone reductase and morphine 6-dehydrogenase were assayed with NADPH and NADP, respectively, as described under Materials and Methods. The ratio given is that of naloxone reductase activity to morphine 6-dehydrogenase activity.

† Obtained from 82 g of liver.

Table 2. Comparison of susceptibilities of naloxone reductase and morphine 6-dehydrogenase to various compounds*

Compound	Conen (mM)	Inhibition (%) Naloxone reductase	Inhibition (%) Morphine 6-dehydrogenase
Pyrazole	1.0	0	0
Barbital	1.0	4	2
Quercitrin	0.1	60	58
$CuSO_4$	0.1	92	96
Lithocholic acid	0.1	98	96
Phenylarsine oxide	0.1	44	50
Prostaglandin E_1	0.1	48	56
Ketamine	0.1	20	40
Naloxone	0.1		38
Morphine	1.0	6	

* The enzyme was preincubated with inhibitor in the presence of NADPH or NADP at 25° for 2 min, and then the reaction was initiated by the addition of naloxone or morphine.

of naloxone reductase and 27% of morphine 6-dehydrogenase were inhibited by 0.1 mM ketamine in 67 mM sodium phosphate buffer, pH 7.4. Naloxone apparently inhibited morphine 6-dehydrogenase at 0.1 mM, whereas morphine gave only a slight inhibition even at 1 mM. Pyrazole, an inhibitor of alcohol dehydrogenase, and barbital, an inhibitor of aldehyde reductase, had no effect on the two enzymes at 1 mM.

Properties of enzyme for naloxone reduction. The preceding results indicated that the reduction of naloxone and the dehydrogenation of morphine are catalyzed by the same enzyme. The molecular weight, isoelectric point, and catalytic properties of the enzyme for the dehydrogenation of morphine were presented in a previous paper [15]. For the reduction of naloxone, the enzyme utilized both NADPH and NADH as cofactor. The pH optima in 67 mM sodium phosphate buffer were 6.8 with NADPH and 6.2 with NADH. Under the optimal pH conditions, the activity with NADPH was about 2.7 times of that with NADH. The apparent K_m values for NADPH and NADH estimated by the Lineweaver–Burk plot [19] were 6.5 and 2.2 μM respectively. The V_{max} values were 1.2 units/mg protein with NADPH and 0.5 unit/mg protein with NADH. The K_m values for naloxone were 0.27 mM with NADPH and 0.44 mM with NADH. The K_m/V_{max} value with NADPH was about one-fourth of that with NADH. Therefore, the reduction of naloxone by the enzyme was more efficiently catalyzed with NADPH than with NADH.

Identification of reaction product of naloxone. When the methanol eluate of the reaction mixture from the Sep-Pak C_{18} cartridge was subjected to TLC, two positive spots with Dragendorff reagent appeared which co-migrated with authentic naloxone (R_f 0.60) and 6α-naloxol (R_f 0.30). The spot corresponding to 6β-naloxol could not be detected. 6α-Naloxol was enzymatically formed, because the spot corresponding to R_f 0.30 was not observed when the incubation was carried out with the enzyme kept for 5 min in boiling water bath or without NADPH in the reaction mixture.

To confirm the result obtained by TLC analysis, the reaction product was also analyzed by GLC. All the samples for GLC analysis were silylated with Tri-Sil Z (Pierce Chemical Co., Rockford, IL) by heating in an oil bath at 90–95° for 2.5 hr. After derivatization, naloxone was revealed at 11.4 min. As shown in Fig. 3A, however, standard samples of both 6α- and 6β-naloxol gave two peaks: 6α-naloxol, a large one at 11.0 min and a smaller one at 14.8 min; 6β-naloxol, a large one at 9.8 min and a smaller one at 16.4 min. Since the peak of naloxone and the large peak of 6α-naloxol were not separated well, the methanol eluate of the reaction mixture from the Sep-Pak C_{18} cartridge was subjected to preparative TLC to eliminate unchanged naloxone. The region containing the reaction product (R_f 0.15–0.40) was scraped off and extracted with methanol. This methanol extract was evaporated to dryness, treated with

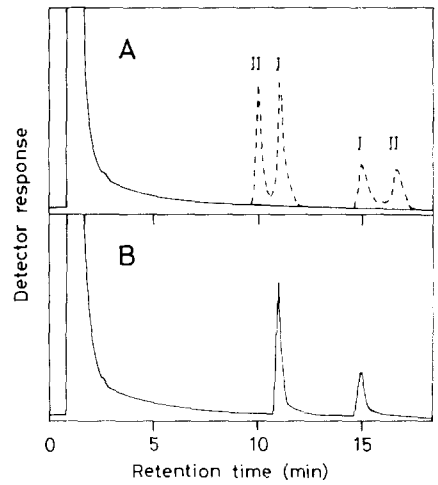

Fig. 3. Gas–liquid chromatogram of reaction mixtures. Preparation of the samples for GLC analysis is described in the text. Solid line in A, incubated with the heat-treated enzyme; dashed line in A, 6α-naloxol (I) and 6β-naloxol (II). B, incubated with the complete system.

Tri-Sil Z, and then injected into the gas chromatograph. As shown in Fig. 3B, the two peaks (retention times at 11.0 and 14.8 min) corresponding to the peaks of standard 6α-naloxol were obtained. As in the case of TLC analysis, 6β-naloxol was not detected.

DISCUSSION

The present study clearly demonstrates that naloxone reductase is identical to morphine 6-dehydrogenase in the guinea-pig liver cytosol, by comparing the elution profiles of the two enzyme activities during purification, the ratios of the two enzyme activities at each purification step, and the stability and susceptibility to inhibitors for the two enzyme activities. In addition, cofactor requirements, optimal pH and kinetic parameters of the enzyme for the reduction of naloxone were clarified. The homogeneity of the enzyme obtained at the final step was confirmed previously by polyacrylamide gel disc electrophoresis and isoelectric focusing with polyacrylamide gel plate, and the enzyme was characterized with respect to molecular weight and certain other parameters [15]. Although the widespread distribution of naloxone reductase among various animal species has been reported, the purification and characterization of the enzyme have not so far, apparently, been published. Accordingly, the present study appears to be the first in which naloxone reductase was purified to a homogeneous protein and characterized extensively.

Recently, Felsted and Bachur [20] reviewed the carbonyl reductases. In this review, the carbonyl reductases were tentatively classified into two groups, aldehyde reductases and ketone reductases. Many similar properties were shared by aldehyde reductases and ketone reductases, but the two enzyme groups were distinguishable from each other by the differences in the susceptibility to inhibitors and substrate specificities. Similar to most of the carbonyl reductases, the present enzyme localized in the cytosol, gave higher activity with NADPH than with NADH, and possessed monomeric low molecular weight (about 29,000 by gel filtration). Naloxone reductase in the guinea-pig liver cytosol was inhibited by quercitrin but not by pyrazole and barbital as proposed for the ketone reductase classified by Felsted and Bachur [20].

The rate of the reduction of naloxone by the enzyme was slightly faster than that of the dehydrogenation of morphine under the optimal pH conditions. In our previous paper [15], it was shown that the enzyme exhibited specificity for morphine analogues having an unsaturated bond at C-7,8 such as nalorphine, normorphine, codeine and ethylmorphine. For the reduction of oxo compounds, naltrexone and oxycodone, which have a C-14 hydroxyl group, as well as naloxone and morphinone were utilized as substrates, but dihydromorphinone and dihydrocodeinone were poor substrates and other aliphatic and aromatic ketones gave negligible or no activity (S. Yamano and S. Toki, unpublished result). Thus, the enzyme seems to exhibit specificity for the oxidoreduction of the 6-hydroxyl group of morphine analogues having an unsaturated bond at C-7,8 and/or a C-14 hydroxyl group.

Roerig *et al.* [11] showed that the guinea-pig liver cytosol contains the activity to form both 6α- and/or 6β-naloxol from naloxone, and 6α-naloxol was a predominant product. Similarly, we confirmed that the soluble fraction and the ammonium sulfate precipitated fraction were capable of forming 6β-naloxol, though in a trace amount as compared to the 6α-naloxol formed. In the study of Roerig *et al.* [11], it was also shown that the peaks of the enzyme activities reducing naloxone to 6α- and 6β-naloxol were precipitated in different fractions on ammonium sulfate fractionation of liver cytosol and only the reduction of naloxone to 6α-naloxol was stimulated by morphine. In the present study, it was confirmed that the purified enzyme is concerned with the stereospecific reduction of naloxone to 6α-naloxol. From this finding together with the results of Roerig *et al.* [11], it appears that 6β-naloxol must be formed by a different enzyme. However, the presence of other naloxone reductases was not indicated in our investigation. Considering the amount of 6β-naloxol formed with the ammonium sulfate precipitated fraction, an unadsorbed protein fraction on the Mātrex green A column chromatography, which showed a very weak activity to reduce naloxone, may participate in the formation of 6β-naloxol from naloxone. However, this minor fraction was not studied further because of its low activity. Except for the Mātrex green A column chromatography step, the enzyme was eluted as a single peak on all the column chromatographies during the purification. From these findings, even though the multiple forms of naloxone reductase could be expected in the guinea-pig liver cytosol, it is evident that naloxol is predominantly produced by the present enzyme.

Although the present enzyme catalyzes the reduction of naloxone to 6α-naloxol, morphine did not exhibit a stimulatory effect on the purified enzyme. Moreover, morphine slightly inhibited at 1 mM where the significant increase of the formation of 6α-naloxol from naloxone was shown with the enzyme preparation obtained by the ammonium sulfate precipitated fraction of liver cytosol by Roerig *et al.* [11]. The reason for this discrepancy is unclear at the present time.

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