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ENZYMATIC CONVERSION OF MORPHINE TO PSEUDOMORPHINE*

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Abstract The interaction of morphine with an enzymatic oxidation reduction system was investigated. It has been reported that morphine is converted to a highly water-soluble metabolite by incubation with horseradish peroxidase (HRP), albumin and H_2O_2 (Misra and Mitchell, *Experientia* 27, 1442 (1971)). After incubating [^{14}C]morphine with HRP and H_2O_2, we detected two radioactive compounds with t.l.c. One compound corresponded to morphine. Using spectral analysis, the other, ^{14}C-labeled compound, was identified as pseudomorphine, a dimerized oxidation product of two molecules of morphine. Using compounds structurally related to morphine, it was determined that: (1) a free phenolic hydroxyl group in position 3 was necessary for the enzymatic oxidation of morphine to pseudomorphine; (2) a carbonyl group in position 6 of the morphinan ring prevents the formation of the dimer; and (3) substitution of other functional groups on the morphine molecule did not affect the peroxidase-catalyzed dimerization. By optimizing reaction conditions, measurable rates of peroxidase-catalyzed pseudomorphine formation were observed at morphine concentrations as low as 5×10^{-7} M. The nature of peroxidase enzymes and the phenolic character of morphine suggest that the formation of pseudomorphine proceeds through a morphine-free radical intermediate.

The interaction of morphine with enzymatic oxidation–reduction systems has been demonstrated by several investigators. Wang and Bain found that incubation of morphine with a reduced pyridine nucleotide and ferricytochrome *c* resulted in the nonenzymatic reduction of the cytochrome *c* [1]. Hosoya and Brody [2] found that incubation of morphine with rat liver homogenate to which cytochrome *c* had been added resulted in the condensation of two morphine molecules to form pseudomorphine. More recent work by Wang and Roerig demonstrated that morphine acts as a catalyst in the transfer of reducing equivalents from a reduced pyridine nucleotide to ferricytochrome *c*, presumably through some reversible oxidation reduction intermediate of morphine [3, 4].

Recently, Misra and Mitchell reported that incubation of morphine with horseradish peroxidase (HRP), $MnCl_2$ or H_2O_2 and albumin resulted in the formation of some highly water-soluble product of morphine [5]. They suggested that a reactive intermediate of morphine was formed which became covalently bound to albumin.

Because of our interest in the interaction of morphine with enzymatic oxidation reduction systems, the present work was designed to study the interaction of HRP and morphine in relation to our previous work with cytochrome *c* and morphine.

MATERIALS AND METHODS

Horseradish peroxidase Type II (125 units/mg) and human serum albumin were purchased from the Sigma Chemical Company, St. Louis, Mo. Morphine sulfate (USP), and morphine (^{14}C, *N*-methyl) hydrochloride (5 μCi/μmole) were obtained from the Mallinckrodt Chemical Company, St. Louis, Mo. All other opiates were obtained from their respective manufacturers and used without further purification.

Incubation of morphine with HRP. In initial experiments, the reaction mixture used was the same as that described by Misra and Mitchell [5]. For most of our studies, however, this reaction mixture was modified and contained 0.041 μmole morphine sulfate, 0.018 mole morphine (^{14}C, *N*-methyl) HCl (1 μCi), 3 units of HRP (0.025 mg protein), 0.18 μmoles H_2O_2 with 0.1 M Tris buffer pH 9.0 to a final volume of 1.0 ml. In some experiments albumin (2.0 mg) was included. The reaction mixture was incubated at 37°. In initial experiments, the reaction mixture was allowed to incubate for a given period of time and then extracted according to the method of Misra and Mitchell to quantify products formed from morphine. We found the results of this procedure to be highly variable. Therefore, the progress of the reaction was monitored either by removing 10 μl portions of the reaction mixture and analyzing them by t.l.c. and liquid scintillation spectrometry or by monitoring the production of fluorescent reaction products.

Thin-layer chromatography. Products from the above reaction were quantified by applying a 10-μl portion of the reaction mixture to Gelman instant t.l.c. sheets (ITLC), Type SG and Type SA. The Type SG chromatogram was developed in butanol–acetic acid water (70:3:10) (system A) and the Type SA chromatogram was developed in pyridine–isopropanol–ammonium hydroxide (90:10:1) (system B). The amounts of unchanged morphine and reaction products were measured by cutting the chromatograms into 1 cm-square strips from 1 cm below the origin to 1 cm above the solvent front. The amount

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of radioactivity in each strip was measured by placing it in 6.5 ml of scintillation fluid and counting on a Model 3330 Packard scintillation spectrometer.

The scintillation fluid consisted of 0.05 g PPO and 4.0 g POPOP dissolved in 1.5 l of toluene.

Fluorometric assay. Once the major reaction product was identified as pseudomorphine, a spectrophotofluorometric assay was used to monitor the progress of the reaction. The reaction mixture was the same as that used for the t.l.c. assay, except that the radioactive morphine was replaced by an equal amount of unlabeled morphine. The reaction was initiated by the addition of the H_2O_2 and the increase in fluorescence caused by pseudomorphine formation was followed on an Aminco Bowman Spectrophotofluorometer with excitation wavelength set at 320 nm and emission wavelength at 440 nm. The increase in fluorescence with time was linear for about the first min of the reaction. Therefore the initial linear rate, in terms of fluorescence unit/sec, was used as a measure of pseudomorphine formation.

Isolation of reaction products. A reaction mixture similar to the above was used except that the amount of unlabeled morphine was increased to 1 μmole/ml. The reaction was initiated by the addition of H_2O_2 and allowed to incubate overnight at room temperature (22°). The precipitate formed was collected by centrifugation, washed with 5 ml of 0.1 M carbonate buffer pH 9.0, centrifuged and washed again with 5 ml of methanol. The resultant precipitate was a snow-white powder which was stored in a vacuum desiccator until analyzed.

Synthesis of pseudomorphine. Pseudomorphine was synthesized using a modification of the method of Bentley and Dyke [6]. Two grams of morphine sulfate were dissolved in 200 ml of 0.4% KOH at 80°, then cooled to room temperature, followed by the addition of 80 ml of potassium ferricyanide (2.31 g/ml) added slowly (50 min) with stirring. The reaction mixture was stirred for an additional 30 min and filtered. The yellow precipitate was washed twice with 50 ml of hot methanol to remove morphine. The off-white precipitate was dissolved in a minimum volume of pyridine and 50% (v/v) water–methanol was added until a slight cloudiness persisted. The solution was then kept at 4° for 24 hr, during which pseudomorphine precipitated as fine white crystals. The crystals were collected by filtration and stored in a desiccator under nitrogen. This material melted at 273–276° with decomposition and the i.r., u.v. and fluorescence spectra were similar to the published spectra for pseudomorphine [6, 7].

Spectral methods of analysis. The products of the action of HRP and H_2O_2 on morphine were isolated as described and analyzed with u.v., i.r. and fluorescent spectroscopy. U.v. spectra were run on a Gilford Model 2400 spectrometer equipped with a Model 2430 scanning attachment. For the i.r. spectrum, the compounds were mixed with KBr and pressed into 1.5-mm pellets. Spectra were run on a Perkin-Elmer Model 467 i.r. spectrophotometer equipped with a beam condenser. All fluorescence studies were performed using an Aminco Bowman Dual Monocrometer spectrophotofluormeter.

RESULTS

Incubation of [^{14}C]morphine with HRP, H_2O_2 and albumin followed by extraction with organic solvents as described by Misra and Mitchell [5] resulted in varying per centages (30–60 per cent) of ^{14}C remaining in the aqueous phase. Because of this variation in results, we altered the analysis procedure and used two different t.l.c. systems to study the products of morphine formed in the reaction. In system A, two major spots were observed for a reaction mixture that had been incubated for 6 hr at 37° and then overnight at room temperature, one spot at R_f 0.1 and another spot at R_f 0.8. The spot at R_f 0.8 corresponded to morphine which was spotted as a standard on each chromatogram. In system B, two spots were observed, one at R_f 0.73 and a second at R_f 0.33. The spot at R_f 0.73 corresponded to the standard morphine spot in this system.

In reaction mixtures separated with either t.l.c. solvent system there was considerable streaking of the spots. It was found that decreasing the H_2O_2 concentration by a factor of 100 from that used by Misra and Mitchell resulted in better spot differentiation. In addition, we found no significant differences between reaction mixtures with and without the albumin. In subsequent studies, therefore, the albumin was omitted.

The amount of the radioactive metabolite of morphine that separated on both chromatography systems A and B (R_f 0.1 and R_f 0.33, respectively) increased with time, as shown in Fig. 1. It can be seen that at 5 hr about 90 per cent of the morphine had been converted. The zero time point shows about 24 per cent conversion of morphine. This is due to the fact that some reaction takes place while the reaction mixture is being spotted and dried on the t.l.c. sheet. In control reactions in which either HRP or H_2O_2 was omitted, only about 5 per cent of the radioactivity was found at the R_f of the metabolite.

Identification of reaction product. In order to identify the product(s) of the action of HRP and H_2O_2 on morphine, it was necessary to isolate sufficient quantities of this material from a reaction mixture. The morphine concentration was increased 10-fold

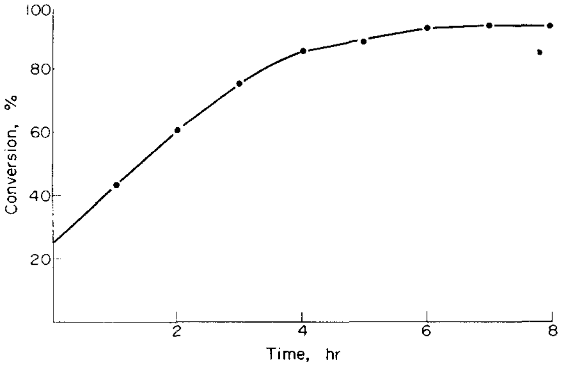

Fig. 1. Per centage of [^{14}C]morphine converted with time to ^{14}C-labeled product by HRP and H_2O_2. Data shown above were obtained using t.l.c. analysis of reaction mixture using chromatography system A. Equivalent results were obtained with system B. Each point is the mean of three separate determinations. Reaction conditions were as described in the text.

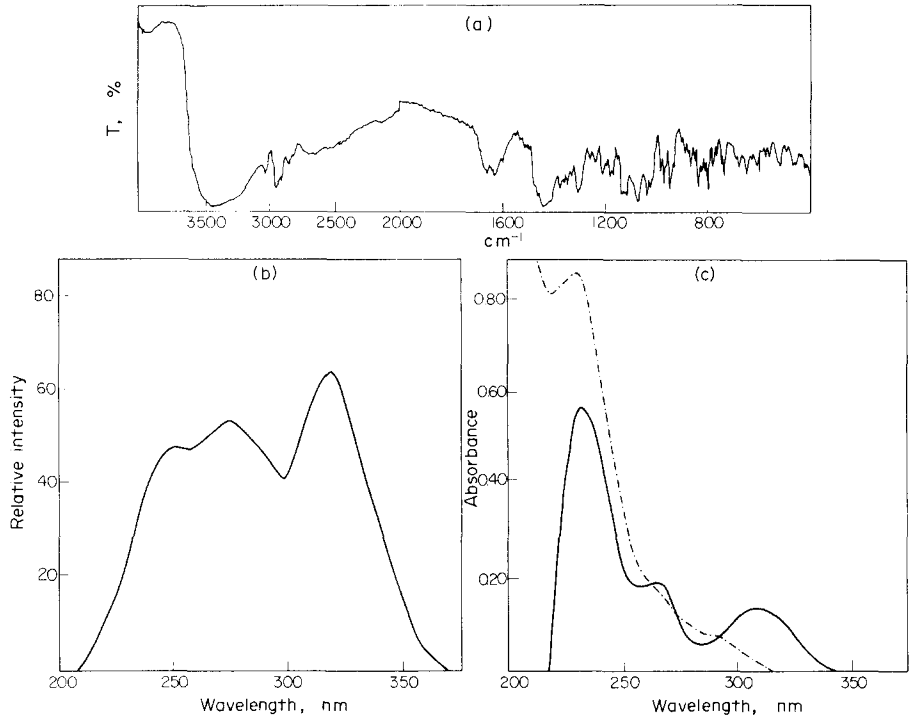

Fig. 2. I.r., fluorescent and u.v. spectra of the product of the action of HRP and H_2O_2 on morphine: (a) I.r. spectrum, per cent transmission vs reciprocal cm (cm^{-1}), (b) fluorescence spectrum in 0.1 M Tris buffer pH 9.0, relative fluorescence intensity vs excitation wavelength (nm). Emission wavelength set at 440 nm, (c) u.v. spectra —·—·— in 0.1 M HCl, and —— in 0.1 M Tris buffer pH 9.0.

(1×10^{-3} M), and the incubation mixture was increased to 5 ml. Under these conditions, a fine white precipitate began to form after about 2 hr at room temperature. This precipitate was purified as previously described (see Methods). Analyses of this material by i.r., u.v. and fluorescent spectrophotometry were performed, and the spectra are shown in Fig. 2. The i.r. spectrum (Fig. 2a) of the reaction product is similar to the published spectrum [7] of pseudomorphine and virtually identical to the i.r. spectrum of pseudomorphine synthesized in our laboratory. The fluorescence spectrum of the reaction product (Fig. 2b) shows the characteristic peaks at 250, 280 and 320 nm for pseudomorphine that were described by Kuferberg *et al.* [8]. In addition, the fluorescence spectrum of the reaction product was the same as that of the synthesized pseudomorphine. The u.v. spectra of the reaction product (Fig. 2c) were run in both acid (0.1 N HCl) and at pH 9.0 (0.1 M Tris). In 0.1 N HCl, a maximum was observed at 231 nm with shoulders at 265 nm and 292 nm. This spectrum is similar to that reported by Holcomb *et al.* for pseudomorphine in 0.05 N HCl [9]. At pH 9.0 the reaction product exhibited maxima at 233, 267 and 310 nm. The u.v. spectra of synthesized pseudomorphine, in both acid and at pH 9.0, were the same as those of the reaction product (Fig. 2c).

In addition to spectral data, further confirmation of the reaction product was obtained from the melting point. The reaction product melted with decomposition between 272 and 275°, as compared to 273 to 276° for the synthesized pseudomorphine and a published value of 276° with decomposition [10].

Optimizing reaction conditions. Since the product of the action of HRP and H_2O_2 on morphine was identified as pseudomorphine, an assay was designed to take advantage of the fluorescence of pseudomorphine (see Materials and Methods). This assay was used for all subsequent studies.

Effect of pH on pseudomorphine formation. The pH of the reaction mixture was varied using different pH buffers at constant ionic strength ($I = 0.1$). The conversion of morphine to pseudomorphine by HRP and H_2O_2 exhibited a pH optimum at 9.0. A second pH optimum was observed at pH 5.5. However, at the lower pH, pseudomorphine was not very stable, resulting in a more non-linear increase in fluorescence with time. As a result, pH 9.0 was used for further studies.

Effect of H_2O_2 *concentration.* The effect of H_2O_2 concentration on the reaction rate was studied by initiating the reaction with 10 μl of various concentrations of H_2O_2. Above an H_2O_2 concentration of 1×10^{-6} M the reaction was essentially zero order with respect to H_2O_2. Above an H_2O_2 concentration of 1×10^{-3} M, however, we did observe some inhibition of pseudomorphine formation. A H_2O_2 concentration of 1×10^{-5} M was selected for our routine reaction mixture.

Effect of morphine concentration. The rate of pseudomorphine formation was measured using morphine in concentrations of 1×10^{-7} M to 1×10^{-3} M. Measurable rates of pseudomorphine formation were observed at morphine concentrations as low as 5×10^{-7} M morphine (0.005 fluorescence units/sec). At higher morphine concentrations (greater than

Table 1. Rate of fluorescence increase of morphine analogs in the presence of HRP and H_2O_2

Compound tested*	Fluorescence units/sec†	Fluorescent maxima in excitation spectra** nm		
Morphine	10.3	250	275	320
Normorphine	0.8	250	275	320
6-Acetylmorphine	0.9	250	275	315
Dihydrodesoxy morphine	0.4	243	273	315
Nalorphine	1.3	250	275	320
EN-2265‡	1.5	250	277	320
Levorphanol	0.3	257		325
Dihydromorphinone	0			
Naloxone	0			
Codeine	0			
Ethylmorphine	0			
Naltrexone	0			
Oxymorphone	0			

* The concentration of all compounds tested was 1×10^{-4} M. Other conditions the same as described in the text.

† Fluorescence units are a per centage of full scale with excitation wavelength at 320 nm and emission at 440 nm.

** Emission wavelength at 440 nm.

‡ EN-2265, *N*-allyl-14-hydroxy-7,8-dihydro normorphine.

10^{-4} M), the reaction rate was less linear with time. In addition, at morphine concentrations above 1×10^{-3} M pseudomorphine precipitated, making the fluorescence assay unusable.

Analogs of morphine. Our previous studies have shown that the functional groups in the morphine molecule are important for its mediation of the NADPH-dependent reduction of cytochrome *c* [1, 3, 4]. Therefore, chemical analogs of morphine were used to determine which functional groups in the morphine molecule were necessary for its enzymatic conversion to pseudomorphine. Shown in Table 1 is a list of compounds that were incubated with HRP and H_2O_2. The reactions were monitored by fluorometry, since condensation of any of these compounds should result in a fluorescent compound analogous to pseudomorphine. Shown are the initial rates of fluorescence increase for each compound. Compounds with a free phenolic hydroxyl group in position 3 (compounds 1–7) exhibited increased fluorescence. In addition, excitation scans resulted in spectra very similar to that of pseudomorphine. The only exception was levorphanol, in which the excitation spectrum consisted of only two peaks, at 257 nm and 325 nm. Compounds 8–13 failed to show any increase in fluorescence when incubated with HRP and H_2O_2. Codeine and ethylmorphine lack the free phenolic hydroxyl group, and dihydromorphinone, naloxone, naltrexone and oxymorphone all have a carbonyl oxygen in position 6 of the morphinan ring. Other changes in the functional groups of the morphinan ring system do not appear to affect the production of fluorescent compounds analogous to pseudomorphine. For example, substitution or elimination of the *N*-methyl group had little effect, since both normorphine and nalorphine formed fluorescent products. In a similar manner, reduction of the double bond between carbons 7 and 8 (nalorphine, dihydrodesoxymorphine, EN-2265) or addition of a hydroxyl group at position 14 of the morphinan ring (EN-2265) did not prevent the formation of a fluorescent product. Addition of the narcotic antagonists naloxone or naltrexone in a concentration of 1×10^{-4} M to a standard reaction mixture containing morphine had no effect on the rate of pseudomorphine formation.

It should be noted that those analogs of morphine which did form fluorescent products in the presence of HRP and H_2O_2 did so at a much slower rate than did morphine. At present, we can offer no explanation for this observation.

DISCUSSION

Incubating [^{14}C]morphine with HRP, albumin and H_2O_2 as described by Misra and Mitchell [5], we found about 30–60 per cent of the morphine remained in the aqueous phase after organic solvent extraction. This finding is in contrast to the 80–90 per cent remaining in the aqueous phase reported by Misra and Mitchell. Using two different t.l.c. systems to analyze the reaction mixture, we found two ^{14}C-labeled spots, one corresponding to morphine and the other to some product of morphine formed during the reaction. In further contrast to the results of Misra and Mitchell, we found that omitting albumin from the reaction did not affect the amount of ^{14}C remaining in the aqueous phase after extraction or the formation of the ^{14}C-labeled product of morphine observed on t.l.c. Using t.l.c., we found that about 90 per cent of the morphine was converted to product in 5 hr (Fig. 1). Special analysis of the reaction product (Fig. 2) identified this compound as pseudomorphine, a condensation product of two molecules of morphine.

The oxidation of morphine to pseudomorphine is catalyzed by a number of other agents such as air [11], u.v. irradiation [12], sunlight [13] and potassium ferricyanide [8, 14]. In fact, the condensation of morphine to pseudomorphine has been reported in almost all reactions involving gentle oxidation conditions for morphine [14, 15]. Pseudomorphine has also been reported to be a morphine metabolite in animals [16], and to be formed from morphine in putrifying tissue [10]. In addition, Hosoya and Brody reported the oxidation of morphine to pseudomorphine by liver homogenates to which cytochrome *c* had been added [4].

Because of the ease with which morphine is oxidized to pseudomorphine, it is not unreasonable that this oxidation is also catalyzed by a peroxidase in the presence of H_2O_2. In general, peroxidase catalyzes a transfer of one reducing equivalent from the substrate to H_2O_2, forming a free radical product of the substrate. The free radical can then dimerize, disproportionate or undergo further oxidation. Guilbault and Hackley have utilized the peroxidase catalyzed dimerization of phenols to develop a fluorescent assay for peroxidase enzymes [17, 18]. These workers found that, in the presence of a peroxidase and H_2O_2, phenols such as homovanillic acid or *p*-hydroxyphenylacetic acid dimerize to a fluorescent product by forming a covalent bond between the aromatic rings at a position *ortho* to the phenolic hydroxyl group. The structure of pseudomorphine suggests that the action of peroxidase on morphine is analogous to its action on other phenolic compounds. Presumably a free radical of morphine is formed by the action of HRP and H_2O_2. This free radical then dimerizes with another morphine molecule to form pseudomorphine. Such a mechanism is consistent with the mechanism proposed by Yeh and Lach for the ferricyanide oxidation of morphine to pseudomorphine [19]. Furthermore, Yeh and Lach suggested that pseudomorphine formation would be favored at a higher pH. The pH optimum in the present study was found to be pH 9.0.

Further support for the formation of a morphine free radical is obtained from the data in Table 1. Essential for a free radical of morphine is the presence of a free phenolic hydroxyl group. With morphine analogs which lack the free phenolic hydroxyl group (codeine and ethylmorphine) no fluorescent products analogous to pseudomorphine were formed.

Wang and Bain found that a free phenolic hydroxyl group was also necessary in the morphine mediated NADH-dependent reduction of cytochrome *c* [1]. In addition, Wang and Roerig found that the oxygen bridge between carbons 4 and 5 of the morphine ring was necessary for this reduction [4]. With the HRP, H_2O_2 system, however, the data indicate that the oxygen bridge is not necessary for the formation of the proposed free radical and subsequent dimerization. As shown in Table 1, levorphanol (3 hydroxy-*N*-methyl morphinan) when incubated with HRP and H_2O_2 did form some fluorescent product, but with a fluorescence spectrum different from pseudomorphine. The fluorescence was, however, dependent on both HRP and H_2O_2 and increased with time, which suggests that a levorphanol analog of pseudomorphine was formed. This difference between the cytochrome *c* system and the HRP system points out that different enzymatic oxidation–reduction systems with which morphine interacts may have different specificities concerning the functional groups in the morphine molecule.

It is interesting to note that no fluorescent products were formed for those morphinans which had a carbonyl group in position 6 (dihydromorphinone, oxymorphone, naloxone, naltrexone). This does not appear to be a function of enzyme specificity, since these compounds fail to produce fluorescent products using the ferricyanide methods of Kuferberg *et al.* [8]. Apparently the carbonyl group in position 6 prevents either free radical formation or dimerization.

Substitution or elimination of other functional groups on the morphine molecule does not appear to affect the HRP catalyzed formation of pseudomorphine. This finding is similar to the specificity found in our earlier work with the transfer of electrons from NADPH to cytochrome *c* [1, 3, 4].

Misra and Mitchell found that addition of the narcotic antagonist nalorphine to their reaction mixture containing [^{14}C]morphine, HRP, H_2O_2 and albumin inhibited the formation of the water-soluble product of morphine [5]. They suggested that this represents a model system for the antagonism of morphine by nalorphine. In the data presented here, however, we found (Table 1) that nalorphine acts as a substrate for HRP and H_2O_2 in a manner like that of morphine. We suggest that these authors observed a simple competition of two substrates (morphine and nalorphine) for the HRP enzyme. In addition, we found that narcotic antagonists such as naloxone or naltrexone did not inhibit pseudomorphine formation.

At present no correlation can be made between the enzymatic oxidation of morphine to pseudomorphine and the pharmacological action of morphine, since the ability of HRP and H_2O_2 to cause the oxidative dimerization of several morphine analogs did not correlate with the pharmacological properties of these compounds. For example, narcotic antagonists nalorphine and EN 2265 did form fluorescent products, while certain analgesics (dihydromorphinone and oxymorphone) and other narcotic antagonists (naloxone and naltrexone) were not oxidized to fluorescent products by HRP and H_2O_2. While the above results do not support a correlation between the HRP-catalyzed oxidation of opiates and their pharmacological properties, the data do not preclude the possibility that such a correlation may exist for some other enzymatic oxidation reduction system that exhibits a specificity different from those systems studied to date. As previously stated, a comparison of the interaction of morphine and its analogs with the cytochrome *c* system [1, 3, 4] and the HRP system demonstrates that different enzymatic oxidation reduction systems can exhibit different specificity concerning the functional groups in the morphine molecule.

It is noteworthy that both of the above reactions have been found to occur at a morphine concentration (5×10^{-7} M) in the range of those estimated in morphine-tolerant animals [1, 4]. Furthermore, the production of a highly reactive free radical metabolite of morphine is interesting in light of the work of Misra and co-workers who have suggested the formation of 2,3 quinone and 2,3 diol metabolites of morphine in rat brain [20–22]. The possibility cannot be overlooked that these metabolites are generated through a free radical mechanism similar to the HRP-catalyzed oxidation of morphine.

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