Biochemical Pharmacology, Vol. 26, pp. 1267–1269. Pergamon Press, 1977. Printed in Great Britain.

SHORT COMMUNICATION

Peroxidase-catalyzed irreversible binding of morphine to protein*

(*Received* 28 *April* 1976; *accepted* 8 *October* 1976)

The reversible binding of morphine to protein has been reported, and opiate binding studies have been used in attempts to identify opiate receptors [1–4]. Recently, Misra and Mitchell [5] reported that, in the presence of horseradish peroxidase (HRP), $MnCl_2$ or H_2O_2 morphine became irreversibly bound to human serum albumin. This conclusion was based on the fact that [^{14}C]morphine could no longer be extracted into an organic solvent after incubation with HRP, $MnCl_2$ or H_2O_2 and human albumin. Using a similar system consisting of morphine, HRP and H_2O_2, our laboratory found that morphine was oxidatively dimerized to pseudomorphine [6]. Furthermore, our findings indicated that the enzymatic conversion of morphine to pseudomorphine involved a free radical intermediate of morphine. It is possible that such a reactive intermediate could react with many other compounds, including protein.

In attempts to study the HRP-catalyzed interaction of morphine with protein, we found that the formation of insoluble pseudomorphine resulted in variable results using the extraction procedure of the previous authors [6]. For this reason, the present work was designed to study the HRP-catalyzed interaction of morphine with protein using two different methods to detect the formation of any morphine–protein complexes. Human serum albumin and synthetic polypeptides were used as model proteins for the study of the morphine–protein interaction.

Morphine[*N*-methyl-^{14}C] hydrochloride (54 μCi/μmole) and unlabeled morphine (USP) were purchased from Mallinckrodt Chemical Co., St. Louis, MO. Horseradish peroxidase (HRP), type II (100 units/mg), crystallized human albumin, poly-L-tyrosine (mol. wt 40,000–100,000), poly-L-histidine (mol. wt 15,000), and poly-L-glutamic acid, type II (mol. wt 15,000–50,000), were obtained from Sigma Chemical Co., St. Louis, MO.

For studies on the interaction of morphine and human albumin, the reaction mixture consisted of 12.5 mg of human albumin, 0.05 μmole [^{14}C]morphine, 0.02 mg HRP, and 0.05 M Tris–HCl buffer, pH 7.5, to a final volume of 5.4 ml. The reaction was initiated by the addition of 50 μl of 0.06% (w/v) H_2O_2. The reaction mixture was incubated in air at 37° with agitation. At 0 time, 30 min and 1, 2, 4, 5, 6 and 24 hr after the addition of H_2O_2, 0.5-ml portions of the reaction mixture were withdrawn and mixed with 0.5 ml of 10% (w/v) trichloroacetic acid (TCA) to precipitate the protein. After centrifugation at 1500 *g* for 5 min, the precipitate was washed twice with 1.0-ml aliquots of 10% TCA. The precipitate was then dissolved in 0.5 ml NCS tissue solubilizer (Amersham–Searle, Arlington Heights, IL), added to 15 ml of liquid scintillation fluid (Aquasol, New England Nuclear) and the amount of ^{14}C determined on a Packard Tri-Carb liquid scintillation spectrometer, model 3330. Controls consisted of two similar reaction mixtures, one in which H_2O_2 was omitted and one in which the albumin was added after 24 hr, followed by an additional 24-hr incubation period.

Polyacrylamide disc gel electrophoresis, according to the method of Clark [7], was also used to demonstrate the irreversible interaction of [^{14}C]morphine and albumin. After the 24-hr incubation period, 0.5-ml portions of the above reaction mixtures were passed through separate 0.4 × 10 cm columns packed with Amberlite XAD-2 resin (Rohm & Haas, Philadelphia, Pa.) to remove as much unbound morphine as possible. The column effluent was monitored by absorption at 280 nm and the fractions containing albumin were pooled. The protein concentration was adjusted to 0.6 mg/ml with the electrophoresis buffer containing 20% (w/v) sucrose.

Fifty ml of the above eluate (30 μg total protein) was applied to the gels. After electrophoresis the gels were stained by soaking for 1 hr in 0.05% Coomassie Brilliant Blue R-250 in 10% TCA. Destaining was accomplished by soaking overnight in 10% TCA. One major protein band and three minor protein bands were observed after staining. The gels were sliced serially with a razor blade into five sections, with no more than one protein band/slice. These slices were dissolved with 0.5 ml of 30% H_2O_2 at 80° for 1 hr and the amount of ^{14}C in each gel slice was determined.

For studies on the interaction of morphine and synthetic polyamino acids, the reaction mixture consisted of 0.01 μmole [^{14}C]morphine HCl, 4 μg HRP, and 0.05 M Tris–HCl buffer to a final volume of 1.09 ml. The amounts of different polyamino acids used were: 2 mg polytyrosine, 1.5 mg polyhistidine, and 2.5 mg polyglutamic acid. The pH of the different reaction mixtures was adjusted to 8.4 for polytyrosine, 7.2 for polyhistidine, and 8.0 for polyglutamic acid to enhance the limited solubility of these polyamino acids. The reactions were initiated by the addition of 10 μl of 0.06% H_2O_2. At 0 time, 30 min, and 1, 2, 3 and 24 hr after initiation, 0.1-ml portions of the reaction mixture were removed. The polytyrosine and polyglutamic acid reaction mixtures were quenched by the addition of 0.2 ml of 1 N HCl to each timed sample, whereas the polyhistidine reaction mixture was quenched by the addition of 0.2 ml of 1 N NaOH to each timed sample. After centrifugation the precipitates were washed twice, dissolved in 0.5 ml NCS tissue solubilizer and the ^{14}C was determined.

The extent to which morphine became bound to albumin in the presence of HRP and H_2O_2 was studied by taking timed samples from the previously described reaction mixture and adding them to equal volumes of 10% TCA. The TCA precipitated the albumin and morphine bound to it, while the unreacted [^{14}C]morphine and pseudomorphine remained in the acid supernatant. Repeated washing of the precipitate insured that all unbound ^{14}C-compounds were removed. Figure 1 shows the percentage of [^{14}C]morphine found in the acid-precipitated protein with time. It can be seen that by 6 hr the morphine binding to albumin approaches a maximum of 46 per cent while in a control reaction mixture in which the H_2O_2 was omitted only about 5 per cent of the radioactivity was associated with the protein precipitate. Since Roerig *et al.* [6] have shown that morphine, in the presence of HRP and H_2O_2, can be converted to pseudomorphine, a second control reaction mixture was run to determine if pseudomorphine would bind to protein. In this experi-

*This work was supported in part by National Institutes on Drug Abuse (NIDA) Grant DA 0114501; Veterans Administration approved Research Project 7460-02.

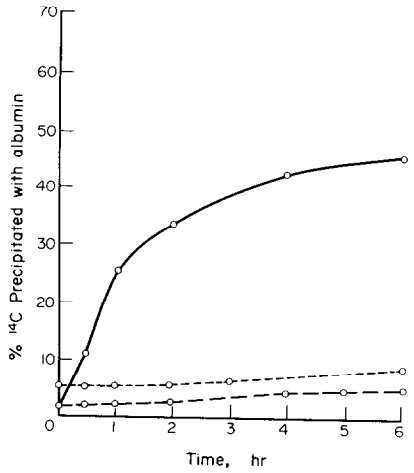

Fig. 1. Percentage of [^{14}C]morphine bound to albumin after precipitation with TCA. Reaction conditions are described in the text. The values represent the per cent of [^{14}C]morphine initially present in the reaction mixture that precipitated after the addition of TCA vs time. Each value is the mean of at least three separate experiments. Key: (— —) represents the complete reaction mixture; (-----) represents a control reaction mixture in which H_2O_2 was omitted; and (· · · · ·) represents a control reaction mixture in which albumin was added after a 24-hr incubation period and samples were taken during an additional 24-hr incubation period.

Table 1. Acrylamide gel electrophoresis of [^{14}C]morphine bound to albumin*

Reaction mixture	Radioactivity in major protein band†
[^{14}C]morphine, HRP, albumin, H_2O_2	1590
[^{14}C]morphine, HRP, albumin, H_2O_2 omitted	129
[^{14}C]morphine, HRP, H_2O_2, albumin added after 24 hr‡	179

* Reaction conditions and preparation of samples are described in the text.

† Each value represents the cpm (^{14}C) found in the gel slices containing the major protein band. Each value is the mean obtained from three separate experiments.

‡ In this control mixture, albumin was added after an initial 24-hr incubation period and samples were taken after an additional 24-hr incubation period.

ment, albumin was omitted from a reaction mixture containing [^{14}C]morphine, HRP and H_2O_2. After 24 hr, albumin was added and the reaction mixture incubated for an additional 24 hr, during which timed samples were removed and treated as described above. As can be seen in Fig. 1, only a small amount (5 per cent) of the ^{14}C was associated with the protein precipitate.

The binding of [^{14}C]morphine to albumin was also studied using polyacrylamide gel electrophoresis to separate the [^{14}C]morphine albumin complex. After staining of the gels, one major and three minor protein bands were observed. The majority of the bound [^{14}C]morphine was associated with the major protein band. Table 1 shows a comparison of the ^{14}C (in cpm) associated with this band and corresponding bands in gels from control reaction mixtures. Approximately 8–10 times as much radioactivity was associated with the major protein band as compared to controls. In the previous experiments, the acid-precipitated albumin from a complete reaction mixture also contained 8–10 times as much [^{14}C]morphine as the precipitated albumin from a control reaction (Table 2). The fact that morphine remains bound to acid-precipitated albumin and cannot be removed by electrophoresis suggests the formation of a strong association between morphine and human albumin. These findings are consistent with those of Misra and Mitchell [5] who found that, after boiling their albumin–morphine complex in 2.4 N HCl, the morphine could not be extracted into an organic solvent. One possible explanation for this strong and apparently irreversible interaction between [^{14}C]morphine and albumin, in the presence of HRP and H_2O_2, is the formation of a covalent bond. This hypothesis is supported by our previous finding that, in the presence of HRP and H_2O_2, morphine was oxidatively dimerized to pseudomorphine which results from the formation of a covalent bond between two molecules of morphine at a position ortho to the phenolic hydroxyl group [6]. We proposed a free radical mechanism similar to the mechanism for the ferricyanide-mediated conversion of morphine to pseudomorphine reported by Yeh and Lack [8]. The peroxidase-catalyzed formation of phenolic free radicals has been studied by Guilbault and Hackley [9, 10] who reported dimerization of several phenolic compounds. A highly reactive free radical of morphine could react with molecules other than itself (for example, with a phenolic amino acid residue such as tyrosine, thus forming a covalent bond with albumin).

The interaction of morphine with specific amino acid residues was studied by incubating [^{14}C]morphine, HRP and H_2O_2 with various polyamino acids. Polyamino acids were used in order to facilitate precipitation of the reaction products. Polytyrosine, polyhistidine, polyglutamic acid, polytryptophan, polyphenylalanine, polyhydroxyproline and polyglycine were studied; however, only the first three were usable, since polytryptophan and polyphenylalanine were insoluble in the reaction mixture, whereas polyhydroxyproline and polyglycine were soluble but could not

Table 2. Per cent of [^{14}C]morphine bound to precipitated albumin or polyamino acid*

Reaction mixture	Albumin† (%)	Polytyrosine† (%)	Polyhistidine† (%)	Polyglutamic acid† (%)
[^{14}C]morphine, HRP albumin, H_2O_2	35.1	72.0	29.5	4.2
[^{14}C]morphine, HRP albumin, H_2O_2 omitted	4.0	2.2	0.9	0.6

* Reaction conditions and preparation of samples are described in the text.

† Each value is the mean of three separate experiments and represents the per cent of the initial ^{14}C in the reaction mixture that precipitated with the albumin or polyamino acid. All values are from samples taken after a 24-hr incubation period.

be precipitated after the reaction. Table 2 shows the results of incubating [^{14}C]morphine, HRP and H_2O_2 with the three different polyamino acids. After a 24-hr incubation period, almost 70 per cent of the ^{14}C was associated with the polytyrosine precipitate and about 30 per cent with the polyhistidine precipitate when compared to controls. Very little, if any, binding of ^{14}C was observed with the polyglutamic acid. These data show that, in the presence of HRP and H_2O_2, morphine becomes strongly bound to specific amino acids. Since tyrosine, like morphine, is a phenolic compound, the fact that the greatest amount of ^{14}C was bound to the polytyrosine further supports formation of a covalent bond mediated through a free radical mechanism.

At present, any correlation of the proposed covalent binding of morphine to protein with some aspect of the pharmacological action of morphine would be purely speculative. Our previous studies on the HRP, H_2O_2 mediated conversion of morphine to pseudomorphine showed that the ability of several structural analogs of morphine to form pseudo-morphine-like dimers did not correlate with the pharmacological actions of these compounds but rather with the presence or absence of certain functional groups on the morphinan ring system [6]. For example, ethylmorphine and codeine, which lack a free phenolic hydroxyl group, did not form analogs of pseudomorphine in the presence of HRP and H_2O_2. Compounds such as dihydromorphinone, oxymorphone, naloxone and naltrexone, which have a keto group in position 6, were also incapable of forming pseudomorphine-like dimers. It would appear that the ability of HRP and H_2O_2 to cause formation of a free radical of the different morphinans and/or the reaction of the free radical with protein would be similarly dependent on the chemical structure of the morphinan and not its pharmacological activity.

It is interesting that Misra *et al.* [11] reported that a ^{14}C-labeled compound remains in the brain of rats for as long as 21 days after a single injection of [^{14}C]morphine. This ^{14}C was not extractable with organic solvents nor sensitive to hydrolysis in strong acids. It is possible that a small amount of morphine becomes covalently bound to some brain protein, mediated by an endogenous peroxidase. However, this study suggests that such a covalent binding of morphine would occur with any protein with the properly exposed amino acids (tyrosine or histidine). The small amount of ^{14}C remaining in the brain, observed by Misra *et al.* [11], could, therefore, be bound to proteins that are not involved in the expression of the pharmacological action of morphine.

Pharmacology Research Laboratory, Veterans Administration Center, Wood (Milwaukee), WI 53193, *and Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI* 53233, *U.S.A.*

MICHAEL J. DEUTSCH
DAVID L. ROERIG
RICHARD I. H. WANG

REFERENCES

1. J. J. Ryan, C. W. Parker and R. C. Williams, *J. Lab. clin. Med.* **80**, 155 (1972).
2. G. D. Olsen, *Clin. Pharmac. Ther.* **17**, 31 (1975).
3. C. B. Pert and S. H. Snyder, *Science, N.Y.* **179**, 1011 (1973).
4. S. H. Snyder and S. Matthysse (Eds), *Neurosci. Res. Prog. Bull.* **13**, 24 (1974).
5. A. L. Misra and C. L. Mitchell, *Experientia* **27**, 1442 (1971).
6. D. L. Roerig, C. J. Reak and R. I. H. Wang, *Biochem. Pharmac.* **25**, 1075 (1976).
7. J. T. Clark, *Ann. N.Y. Acad. Sci.* **121**, 428 (1964).
8. S. Y. Yeh and J. L. Lack, *J. pharm. Sci.* **50**, 35 (1961).
9. G. G. Guilbault and E. Hackley, *Annls Chem.* **40**, 190 (1968).
10. G. G. Guilbault and E. Hackley, *Annls Chem.* **40**, 1256 (1968).
11. A. L. Misra, C. L. Mitchell and L. A. Woods, *Nature, Lond.* **232**, 48 (1971).