Biochemical Pharmacology, Vol. 23, pp. 3163–3171. Pergamon Press, 1974. Printed in Great Britain.

MEDIATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE DEPENDENT REDUCTION OF CYTOCHROME c BY MORPHINE

RICHARD I. H. WANG and DAVID L. ROERIG

Pharmacology Research Laboratory, Clinical Pharmacology Service, Veterans Administration Center, Wood, Wis. 53193, U.S.A.

(*Received* 10 *December* 1973; *accepted* 22 *March* 1974)

Abstract—The ability of morphine and other analgesics to mediate a transfer of electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to cytochrome c was investigated. This phenomenon was first reported by Wang and Bain (*J. Pharmac. exp. Ther.*, **108**, 354, 1953), and we have confirmed their findings and provided further insight into this unusual action of morphine. This electron transfer was studied by following the reduction of cytochrome c and it was found that: (1) in order to mediate the transfer of electrons, the compound studied must be a morphinan with a free phenolic hydroxyl group in the 3 position and an oxygen bridge between positions 4 and 5; (2) substitution or rearrangement of other functional groups on the morphinan molecule did not greatly affect its ability to mediate the electron transfer; (3) by optimizing reaction condition (pH and purification of the morphine), we observed measurable rates of cytochrome c reduction (0·002 A/min) at morphine concentrations as low as $4{\cdot}2 \times 10^{-7}$ M; and (4) preliminary evidence indicates that morphine acts catalytically in mediating the electron transfer. Since narcotic analgesics of the opioid type such as methadone, pentazocine and 3-hydroxy-*N*-methyl morphinan did not mediate the electron transfer, it appears that no correlation exists between this electron transferring action and the mechanism of analgesia in general. This does not, however, preclude that a correlation could exist between this electron transferring action and some other aspect of the pharmacological action of opiates.

THE ABILITY of cytochromes to act as electron acceptors for reduced pyridine nucleotides in the presence of certain flavoproteins is well recognized. The reduction of cytochrome c by specific flavoproteins has been studied extensively, and this hemoprotein has been used as an artificial electron acceptor for a number of different reductases.[1–3] Williams and Kamin,[4] for example, have described a microsomal reductase that would catalyze the following reaction:

$$\text{NADPH} + \text{H}^+ + 2\,\text{cyt-c}^{3+} \xrightarrow{\text{reductase}} \text{NADP} + \text{cyt-c}^{2+} + 2\,\text{H}^+.$$

Wang and Bain,[5] however, while studying the effect of analgesics on various brain enzymes, observed a similar NADH-dependent reduction of cytochrome c in the absence of any reductase enzymes. They determined that this reduction was dependent on the presence of morphine and that through some mechanism morphine was mediating a transfer of electrons from NADH to ferricytochrome c. Wang and Bain termed this phenomenon the "electron transferring action of morphine." It is interesting to note that, except for a brief mention by Hosoya and Brody,[6] the report of this phenomenon has gone virtually unnoticed.

The relationship of this electron transferring action of morphine to any biologic effect of morphine is not yet known. However, because other attempts have not been successful in providing insight into the mechanism of action of morphine and, due to the fact that such a mechanism is ultimately biochemical in nature, all facets of the interaction of morphine in the biological system should be investigated. For this reason, we have re-examined and expanded the original work of Wang and Bain to provide further insight into the mechanism by which morphine mediates a transfer of electrons from NADPH to cytochrome c.

MATERIALS AND METHODS

Cytochrome c (horse heart, type IV) and NADPH were purchased from Sigma Chemical Co. Morphine (USP) and morphine (*N*-methyl-^{14}C) HCl (3·65 mCi/mole) were obtained from Mallinckrodt Chemical Co. All other drugs studied were obtained from their manufacturers. The radioimmunoassay used for morphine quantitation was Abuscreen®, supplied by Roche Diagnostics, Nutley, N.J. All other chemicals were reagent grade.

Reduction of cytochrome c. The reduction of ferricytochrome c was measured spectrophotometrically by following the rate of increase in absorbance at 550 nm, using an assay mixture similar to that of Omura and Takesu.[7] The 1·0-ml assay contained 0·2 ml cytochrome c (0·88 mg), 0·1 μmole NADPH, 0·7 ml of 0·1 M Tris–HCl, pH 8·9, and 0·1 ml of different concentrations of morphine HCl in the Tris–HCl buffer. The reaction was initiated by the addition of the NADPH in 0·01 ml of 0·1 M pH 10·5 carbonate buffer. All reactions were carried out at 22°. The increase in absorbance with time was recorded on a Gilford 2400-N spectrophotometer. Since the increase in absorbance was linear with time for only the initial 10 per cent of the absorbance change, this initial rate was used as the measure of the rate of reduction.

Purification of morphine. Morphine sulfate (USP) was purified using preparative thin-layer chromatography. Morphine sulfate (0·01 M) in methanol was spotted repeatedly across the width of Gelman type SG instant thin-layer chromatography media until 0·5 ml had been applied. The chromatogram was dried and developed in a butanol–acetic acid–water (35:3:10) solvent system A to a solvent height of 12 cm. To locate the morphine, a 2-cm wide strip cut from the side of the chromatogram was sprayed with iodoplatinate. The area on the chromatogram corresponding to the morphine spot was cut out and eluted with three 5-ml aliquots of methanol. The methanol eluate was taken to dryness under a stream of nitrogen and dissolved in 1 ml of 0·01 M HCl. These operations were carried out in a darkened room under a nitrogen atmosphere. The purity of the morphine was checked by thin-layer chromatography in two alternate solvent systems: system B, citric acid–butanol–water (0·48 g: 87:13) and system C, pure methanol. Morphine purified in this manner was used within 2 days after preparation. *N*-methyl-^{14}C morphine was purified by the same method.

Quantitation of purified morphine. Because of the small quantities of morphine purified at one time, the concentration of the purified morphine was measured using radioimmunoassay. The assay mixture consisted of 0·4 ml morphine specific antibody and ^{3}H-dihydromorphine solution to which 0·1 ml of a 1:10,000 dilution of the purified morphine was added. This mixture was allowed to stand at room temperature for 1 hr, then 0·5 ml of saturated $(NH_4)_2SO_4$ was added to precipitate the anti-

body–morphine complex. After centrifugation at 2000 *g* for 5 min, 0·5 ml of the supernatant was added to 10 ml Bray's solution and counted in a Packard model 3380 liquid scintillation counter. A standard curve was prepared using morphine HCl in concentrations of 0–50 ng/ml.

RESULTS

Mediation of NADPH-dependent reduction of cytochrome c by morphine. Initial experiments were conducted to determine the magnitude of the effect of morphine on the reduction of cytochrome c in the presence of NADPH. The original investigations of Wang and Bain[5] were reproduced using NADPH instead of NADH because of the greater stability of NADPH in solution and the absence of potential inhibitor found in NADH preparations.[8] The USP grade morphine used initially gave a slight nonspecific reduction of cytochrome c, which was found to be due to other substances present in the morphine. As a result, small quantities (3–5 mg) of morphine were purified, as described in Materials and Methods, prior to use. Figure 1 shows the effect of purified morphine concentration on the NADPH-dependent reduction of cytochrome c. Not shown in Fig. 1 are the highest and lowest concentrations of morphine studied. At a morphine concentration of $8{\cdot}4 \times 10^{-4}$ M, a rate of reduction of 0·3 A/min was observed. At a morphine concentration as low as $4{\cdot}2 \times 10^{-7}$ M, the rate of reduction was found to be 0·002 A/min (Table 1).

Effect of pH. The effect of pH on the NADPH-dependent reduction of cytochrome c in the presence of morphine was determined. The results of this experiment are shown in Fig. 2. The reduction shows a definite pH optimum at pH 8·9. Above this pH, precipitation of the morphine was observed in experiments where high concentrations of morphine were used. Since phosphate or Tris–HCl buffers at the same pH gave the same rate of reduction, the reaction appears to be independent of the type of buffer ion employed. The reduction was also independent of ionic strength (studied up to an ionic strength of 0·5).

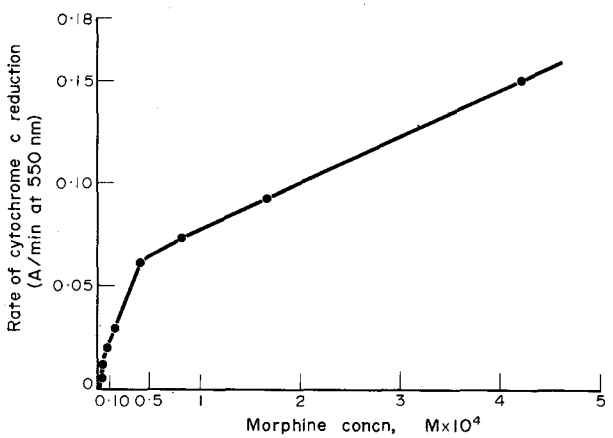

FIG. 1. Effect of morphine concentration on the rate of NADPH-dependent reduction of cytochrome c. Purified morphine was used and the concentration of the morphine was determined using radioimmunoassay. The assay was performed as described in the text. The highest ($8{\cdot}4 \times 10^{-4}$ M) and the lowest ($4{\cdot}2 \times 10^{-7}$ M) morphine concentrations used are shown in Table 1. Each point is the mean obtained from triplicate assays.

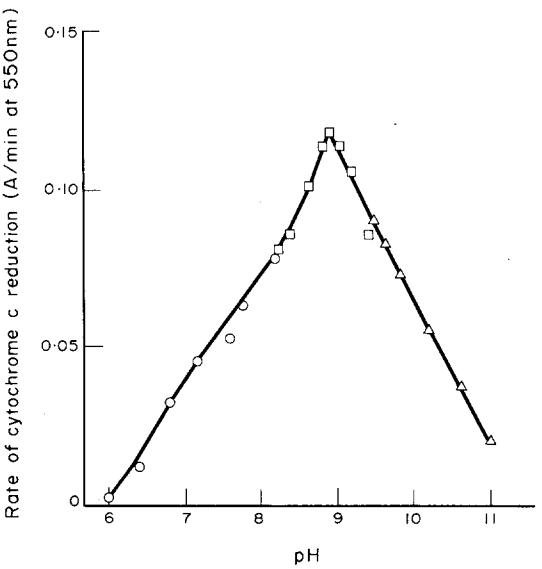

FIG. 2. Effect of pH on NADPH-dependent reduction of cytochrome c. The assay was performed as described in the text. The ○, □ and △ denote the use of phosphate, Tris and carbonate buffers respectively. All buffers were 0·1 ionic strength. Purified morphine concentration was 3×10^{-4} M. Duplicate assays using phosphate and Tris buffers and Tris and carbonate buffers were performed at pH 8·2 and 9·4 respectively.

Studies of the possible role of morphine. Preliminary studies on the role of morphine in mediating a transfer of electrons from NADPH to cytochrome c were undertaken to determine if morphine was acting catalytically or as a reactant. Purified ^{14}C-morphine HCl (0·8 μCi, 3·65 μCi/mole) was incubated with NADPH and cytochrome c, as described. The reaction was followed to completion spectrophotometrically (total absorbance change 0·77 A). A 20-μl portion of the reaction mixture and appropriate controls were applied to a thin-layer chromatogram and developed in solvent systems A, B and C. The developed chromatograms were placed on Kodak RP® X-ray film and exposed for 1 week. Spots containing as little as 0·1 nmole ^{14}C-morphine could easily be visualized using a 1-week exposure of the chromatogram on the X-ray film. This represents a sensitivity of less than 3 per cent of the total ^{14}C-morphine applied to the chromatogram. Figure 3 shows a typical film from such an experiment. The standard morphine solution and reaction mixture gave at least two spots, one at the origin and the other corresponding to morphine. A third spot was detectable in the reaction mixture and in the control reaction that contained morphine and cytochrome c but no NADPH. In this control reaction, no reduction of cytochrome c had taken place. This third spot was present as a thin band just above the spot at the origin and was observed with all three solvent systems.

Effect of morphine analogs. In order to determine which functional groups of morphine are important in the morphine-mediated transfer of electrons from NADPH to cytochrome c, several compounds which were structurally related to morphine were tested. Table 1 summarizes the results of this experiment. It can be seen that compounds which have a free phenolic hydroxyl group at position 3 and an oxygen

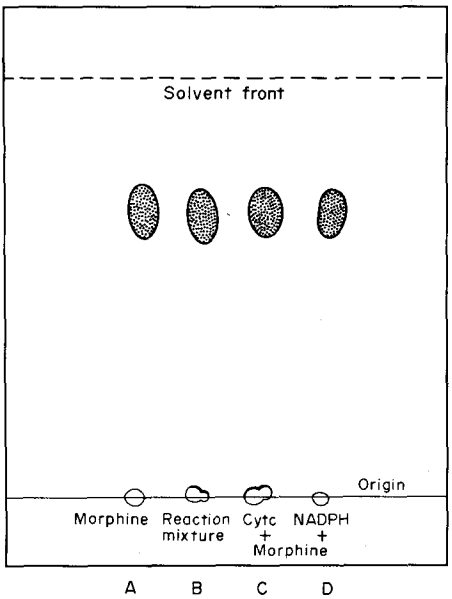

FIG. 3. Typical radioautogram of ^{14}C-morphine incubated with NADPH and cytochrome c. Chromatogram was developed in solvent system B (citric acid–butanol–water, 0·48 g:87:13) and exposed to X-ray film for 1 week. (A) Purified ^{14}C-morphine standard; (B) complete reaction mixture containing ^{14}C-morphine, NADPH and cytochrome c as described in the text; (C) control reaction mixture containing only ^{14}C-morphine and cytochrome c; and (D) control reaction mixture containing ^{14}C-morphine and NADPH.

TABLE 1. EFFECT OF MORPHINE ANALOGS ON THE NADPH-DEPENDENT REDUCTION OF CYTOCHROME C

Compound tested	Concn (moles/l)	Rate of reduction* (A/min)
Morphine	$4{\cdot}2 \times 10^{-7}$	0·002
Morphine	$8{\cdot}4 \times 10^{-4}$	0·30
Normorphine	1×10^{-3}	0·118
Oxymorphone	1×10^{-3}	0·071
6-Acetylmorphine	1×10^{-3}	0·061
Dihydromorphine	1×10^{-3}	0·165
Naloxone†	1×10^{-3}	0·162
EN-2265‡	1×10^{-3}	0·125
EN-1639-A§	1×10^{-3}	0·104
Apomorphine‖	1×10^{-6}	0·10
3-Hydroxy-*N*-methyl morphinan	1×10^{-3}	No reaction
Codeine	1×10^{-3}	No reaction
Pentazocine	1×10^{-3}	No reaction
Meperidine	1×10^{-4}	No reaction
Methadone	1×10^{-3}	No reaction

* Rate of reduction of cytochrome c in the presence of NADPH is expressed as change in absorbance at 550 nm/min.

† Naloxone, *N*-allyl-14-hydroxy-7,8-dihydro normorphanone.

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

§ EN-1639-A, *N*-cyclopropyl-methyl-7,8-dihydro-14-hydroxy normorphine.

‖ Rate of reduction of cytochrome c by apomorphine was measured in the absence of NADPH.

bridge between positions 4 and 5 of the morphinan ring system exhibit the ability to mediate the electron transfer. Substitution or rearrangement of other functional groups in the molecule does not appear to be important. For example, a keto group in the 6 position or reduction of the double bond between positions 7 and 8 has little effect. Similarly, substitution or elimination of the *N*-methyl group does not affect the reduction. One curious result was obtained with apomorphine. When this compound was added to the cytochrome c, immediate reduction took place in the absence of NADPH.

DISCUSSION

In their original report of the ability of morphine to mediate a transfer of electrons from NADH to cytochrome c, Wang and Bain[5] found rates of reduction of cytochrome c which ranged from 0·005 A/min at a morphine concentration of $5{\cdot}0 \times 10^{-4}$ M to a high of 0·015 A/min at 1×10^{-3} M morphine. Assuming equal tissue distribution, these authors estimated that even in the tolerant animal the concentration of morphine would be only about 1×10^{-7} to 1×10^{-6} M, far below the concentrations used in their work *in vitro*. In the present study, however, we have optimized conditions and have measured a rate of cytochrome c reduction of 0·002 A/min at a morphine concentration as low as $4{\cdot}2 \times 10^{-7}$ M at a pH of 8·9. It is difficult to assess the significance of this result, since it was obtained at a pH well above normal physiological pH. However, it should be noted from Fig. 2 that at a pH of 7·4 the rate of reduction is about three times lower than at pH 8·9. Relating this to Fig. 1, it can be seen that at pH 7·4 a morphine concentration of 10^{-6} M would still mediate the NADPH reduction of cytochrome c at a measurable rate (approximately 0·004 A/min). This is still within the physiological limits estimated by Wang and Bain, and represents a decrease of two orders of magnitude in the lowest concentration of morphine at which the NADPH-dependent reduction of cytochrome c can be measured. Furthermore, as shown in Table 1, the highest rate of reduction observed (0·3 A/min at $8{\cdot}4 \times 10^{-4}$ M morphine) is about 20 times greater than that found in the earlier work.

The reasons for the large differences between the results presented here and those of the previous investigators are not completely clear. However, these differences may be due, at least in part, to several differences in experimental conditions. In the present experiments, morphine was purified in order to eliminate small amounts of impurities which reduced cytochrome c directly. As a result, we found that the ability of morphine to mediate this reduction increased about 2-fold after purification. In addition, Wang and Bain used an assay mixture at pH 7·4, whereas a pH of 8·9 was used in these studies. As previously mentioned, the rate of reduction is about three times higher at pH 8·9 than at pH 7·4. The present study also employed NADPH instead of NADH as a source of reducing equivalents. Finally, the use of a more modern instrumentation, such as a recording spectrophotometer with scale expansion, has greatly facilitated measuring the low initial rates of reduction involved.

In agreement with the original work of Wang and Bain,[5] our studies with morphine analogs have demonstrated which arrangements of certain functional groups within the morphinan ring system are necessary for the mediation of a transfer of electrons from NADPH to cytochrome c. As shown in Table 1, compounds like codeine which lack a free phenolic hydroxyl group in position 3 do not mediate the

reduction of cytochrome c. In a similar manner, a compound like 3-hydroxyl-*N*-methyl morphinan, in which the oxygen bridge between positions 4 and 5 is absent, does not mediate the reduction. Table 1 also indicates that substitution or rearrangement of other groups on the morphinan ring system does not greatly affect the ability of the molecule to mediate the NADPH-dependent reduction of cytochrome c. For example, hydromorphone or oxymorphone with a keto group in the 6 position and a single bond between positions 7 and 8 has a capacity to effect the reduction similar to that of morphine. Substitution or elimination of the *N*-methyl group appears to have little effect since naloxone, EN-2265, EN-1639-A and normorphine possess activity similar to that of morphine. Also, a hydroxyl group in the 14 position (naloxone, EN-2265, EN-1639-A and oxymorphone) does not alter the reducing capacity.

One compound that was found to act quite differently from all the other morphine analogs was apomorphine. This compound was found to reduce cytochrome c directly in the absence of NADPH. In fact, the apomorphine concentration had to be reduced to 1×10^{-6} M before the rate of reduction was low enough to be measured spectrophotometrically. From this finding, it appears that apomorphine is acting as a strong reducing agent. Apomorphine seems to have no effect on the transfer of electrons between NADPH and cytochrome c, but is itself the electron donor. This situation is similar to that reported by Aviram[9] in his studies on the auto-reduction of cytochrome c in glycine–NaOH buffer. He found that the auto-reduction was actually due to the reduction of ferricytochrome c by the amino group of glycine, with concomitant formation of glyoxylic acid.

It is interesting that USP grade morphine did contain a small amount of impurity that reduced cytochrome c directly. Those morphine analogs capable of mediating the transfer of electrons from NADPH to cytochrome c would also cause a slight reduction of cytochrome c in the absence of NADPH. Perhaps the impurity in these compounds is structurally similar to apomorphine. Further studies are now in progress concerning this phenomenon.

It should be noted that, with the exception of morphine, the compounds tested (Table 1) were not purified prior to use. The purity and degree of hydration of these compounds are unknown. This fact could explain why they gave rates of NADPH-dependent cytochrome c reduction somewhat lower than those found with equimolar amounts of purified morphine, since we found that purification of morphine results in about a 2-fold increase in its ability to mediate the reduction.

Some preliminary studies were performed to investigate the role of morphine in the NADPH-dependent reduction of cytochrome c. This was accomplished by using ^{14}C-labeled morphine in the reaction mixture and determining by thin-layer chromatography and radioautography if any metabolites of morphine were formed during the reaction. As shown in Fig. 3, three spots were observed on the radioautogram when a portion of a reaction mixture containing ^{14}C-morphine, NADPH and cytochrome c was chromatographed. The spot at R_f 0·75 corresponded to morphine. The ^{14}C-labeled spot at the origin of the chromatogram does not appear to be a product of the reaction, since it was found in all controls including the control mixture containing only ^{14}C-morphine in buffer. A third spot was found slightly above the spot at the origin in the complete reaction mixture and in a control reaction mixture containing cytochrome c but no NADPH; in the latter case, no reduction had taken place. Since morphine has been shown to be adsorbed onto protein,[10] the third

spot is probably due to a nonspecific interaction between morphine and cytochrome c which results in a very slow-moving component. By cutting out the radioactive areas of the chromatogram and counting them by liquid scintillation, we found that the ^{14}C-morphine spot routinely accounted for greater than 95 per cent of the radioactivity applied to the chromatogram. Similar results were obtained with all three solvent systems except that the R_f of morphine differed from one system to another.

Hosoya and Brody[6] reported that incubation of morphine with cytochrome c and rat liver homogenate resulted in the production of a a fluorescent compound that was chromatographically identical to pseudomorphine. In our studies, no such fluorescent material was observed when purified morphine was incubated with NADPH and cytochrome c. Furthermore, if morphine were being converted to some other compound by the NADPH-dependent reduction of cytochrome c, one would not expect ^{14}C-labeled morphine to account for such a high percentage of radioactivity in the reaction mixture. For example, based on the E_{550} (reduced-oxidized) of $2{\cdot}1 \times 10^4$ cm^2/mole for cytochrome c[11] and a total absorbance change of 0·77 A (550 nm) for the reaction, one calculates that $3{\cdot}7 \times 10^{-8}$ moles of cytochrome c were reduced in the 1-ml reaction mixture. If morphine, for instance, were oxidized by the reaction and assuming 1 mole of cytochrome reduced/mole of morphine oxidized, $3{\cdot}7 \times 10^{-8}$ moles of morphine would be oxidized. This would represent about 17 per cent of the ^{14}C-morphine present in the reaction mixture. It is obvious that this situation does not exist, since morphine accounted for at least 95 per cent of the radioactivity in the reaction mixture after the reduction of cytochrome c. Even if one assumed 2 moles of cytochrome c reduced/mole of morphine metabolized, the amount of morphine converted to some other compound would be about 9 per cent of the total morphine. This would require that no more than about 85 per cent of the radioactivity appear in the ^{14}C-morphine spot, which was not the case. The data, therefore, suggest to us that morphine is not a reactant but rather a catalyst which mediates a transfer of electrons from NADPH to cytochrome c.

The possible mechanisms by which morphine mediates the transfer of electrons from NADPH to cytochrome c may be quite interesting. Since both the free phenolic hydroxyl group and the oxygen bridge of morphine are necessary for the electron transferring phenomenon to occur, it is reasonable to speculate that the reaction is mediated through a reversible electronic rearrangement involving these groups and the aromatic ring. However, more in depth kinetic and model compound studies are needed to elucidate the actual mechanism.

While Table 1 confirms the importance of the functional groups involved and Fig. 3 indicates that morphine is acting catalytically in the transfer of electrons from NADPH to cytochrome c, these data do not establish a correlation between analgesic activity and the ability of a compound to mediate this reduction. In fact, the data suggest that no correlation exists for analgesia in general, since narcotic analgesics of the opioid type such as methadone, meperidine and pentazocine would not mediate a transfer of electrons from NADPH to cytochrome c. However, it may still be possible that a correlation exists between this electron transferring action and the mechanism of some other aspect of the pharmacological action of opiates. The fact that codeine and heroin[5] did not mediate the electron transfer does not preclude the possibility of such a correlation, since these compounds have been shown to be meta-

bolized to morphine[12] and 6-acetyl morphine[13] respectively. As is shown in Table 1, both metabolites are very active in mediating the electron transfer.

It is interesting that compounds such as naloxone, EN-2265 and EN-1639-A, which are structurally classified as opiates and mediate the electron transfer, are themselves narcotic antagonists. This finding would support a hypothesis that the electron transferring action of the other opiates is not related to the mechanism of opiate analgesia. This is further supported by the fact that naloxone and EN-1639-A have been shown to be nearly pure narcotic antagonists in man, although some agonist activity has been observed in pigeons.[14,15]

In conclusion, from these experiments it appears that opiates such as morphine or its analogs with a free hydroxyl group in the 3 position and an oxygen bridge between positions 4 and 5 act catalytically to mediate a transfer of electrons from NADPH to cytochrome c. How this is related to the action of morphine cannot be concluded from these data, but possible relationships are now under investigation. The fact that this electron transfer can be observed at morphine concentrations in the range of 1×10^{-7} to 1×10^{-6} M does suggest, however, that it could have some pharmacological importance. Of greatest interest, however, is that morphine does catalyze a transfer of electrons. Although only the NADPH-cytochrome c system has been studied, it is possible that morphine can mediate a transfer of electrons in other enzymatic electron transport systems, a phenomenon that may provide insight into the mechanism of action of morphine on a number of enzyme systems.

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