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EVIDENCE FOR A NEW METABOLITE OF MORPHINE-*N*-METHYL-^{14}C IN THE RAT

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Abstract—Evidence has been presented for the formation *in vivo* in the rat of a new urinary metabolite of morphine, to which 2,3-dihydrodiol structure has been tentatively assigned. *In vitro*, rat brain and liver homogenates were shown to produce a 2,3-catechol type of metabolite by aromatic hydroxylation of morphine. Small amounts of morphine *N*-oxide and normorphine were also identified as metabolites *in vitro* by liver homogenates. Sequential oxidation of morphine by alkaline ferricyanide and hydrogen peroxide and Cu^{2+} has been shown to produce a zwitterionic 2,3-quinone, whose chromatographic properties appeared similar to those of the hydroxylated metabolite formed *in vitro* by rat brain and liver homogenates.

ALTHOUGH much information is available on the biotransformation of morphine in various animal species, including some in man, the metabolic fate of this drug has not been completely established in the form of metabolites of known structure. Glucuronide conjugation[1–5] at the 3- and 6-position, 3-ethereal sulfate conjugation,[6–8] *N*-dealkylation,[9–12] 3-methylation,[13] and *N*-oxide formation[14] constitute the known metabolic pathways[15] for morphine in various animal species.

A recent study[16] on the persistence of morphine in the central nervous system of rats after a subcutaneous injection of morphine provided evidence for the formation of a catechol type of metabolite of morphine in the CNS of rats. Sensitive methods for the estimation of morphine-*N*-methyl-^{14}C oxide[17] and pseudomorphine-^{3}H[18] in biological materials ruled out the formation of these two compounds as metabolites of morphine in the rat brain. Liver microsomes *in vitro* in the presence of NADP, glucose 6-phosphate, catechol-*O*-methyl transferase and *S*-adenosyl methionine have been shown[19] to convert a small quantity of morphine (1·2 nmoles/g liver) to catechol and a methylated catechol type derivative.

This report provides evidence for the formation of new metabolites of morphine *in vivo* in the rat and *in vitro* by liver and brain homogenates.

MATERIALS AND METHODS

Male Sprague–Dawley rats (150–200 g) were injected subcutaneously with 10 mg/kg of morphine-*N*-methyl-^{14}C (specific activity, 1·94 μCi/mg), and urine and feces collected in Delmar Roth metabolism cages up to 48 hr. Urine from ten different rats was analyzed for free and conjugated morphine. Free morphine was estimated by extracting 2-ml aliquots of urine containing 1 ml of nonradioactive morphine carrier (1 mg/ml), adjusted to pH 9 with dil. NaOH, buffered with 2 ml of 40% K_2HPO_4 solution and extracted by shaking for 30 min with 15 ml ethylene dichloride

containing 30% by volume of *n*-amyl alcohol. The solution was centrifuged, aqueous phase removed by aspiration, and organic phase washed with 4 ml of 4% K_2HPO_4. The residue from 10-ml aliquots of the organic phase evaporated in counting vials on a Fisher slide warmer was dissolved in 0·8 ml *n*-amyl alcohol. Ten ml toluene–phosphor was added to each sample and radioactivity counted in a Nuclear Chicago Mark II liquid scintillation spectrometer. The radioactivity was corrected for quenching using toluene-^{14}C as an internal standard.

Aliquots of urine were autoclaved with 20% by volume of conc. HCl for 1 hr at 15–20 lb of pressure, samples were cooled, adjusted to pH 9, and extracted as above to obtain total morphine (free and acid-hydrolyzable conjugate).

Chromatographic studies. Pooled urine was centrifuged to separate the insoluble matter, and passed through a column of Amberlite XAD-2 (25 × 350 mm) resin.* The column was washed with water and eluted with 300 ml methanol containing 10 ml conc. NH_4OH. The residue from the methanol eluate was evaporated to dryness *in vacuo*, and triturated with methanol to separate soluble and insoluble portions for further manipulations.

Methanol-soluble fraction from Amberlite XAD-2 column. This fraction was chromatographed on Gelman ITLC† (Silica gel) with solvent system *n*-butanol–acetic acid–water (35:3:10, v/v) [Fig. 1(1)] and the radiochromatogram scanned.‡ Two major fractions were obtained: (a) polar metabolites consisting of mixed conjugates of morphine (R_f 0·44), and (b) mixed free bases (R_f 0·80).

Mixed free bases fraction. This fraction was eluted from ITLC large sheets (20 × 20 cm) with aqueous methanol and rerun on ITLC with *n*-butanol–acetic acid–water (35:3:10, v/v), when a single peak of radioactivity (R_f 0·80) and a single colored band were obtained with the iodoplatinate spray reagent [Fig. 1(2)]. ITLC with ethyl acetate–methanol–conc. ammonia (17:2:1, v/v) produced two bands of radioactivity; one, R_f 0·0 (approximately 5–6 per cent of the injected dose), and another, R_f 0·90 (morphine) [Fig. 1(3)]. A nonradioactive band, R_f 0·70 to 0·74, positive to iodoplatinate and ninhydrin reagent, was also visualized and shown to be due to normorphine by co-chromatography in several chromatographic solvent systems. The compound, R_f 0·0 on TLC in solvent system *n*-butanol–acetic acid–water (35:3:10, v/v), gave a peak of radioactivity with R_f 0·90 [Fig. 1(4)].

The mixed free bases fraction from ITLC, chromatographed on Whatman 3MM paper with solvent system *n*-butanol–acetic acid–water (4:1:2, v/v), gave a major peak of radioactivity, R_f 0·68, minor peak, 3–5 per cent, R_f 0·48 [Fig. 1(5)], and with solvent system *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v), a major peak of radioactivity, R_f 0·75, and minor ones with lower R_f [Fig. 1(6)].

Larger quantities of compound, R_f 0·0 [Fig. 1(3)] were separated on large ITLC sheets (20 × 20 cm) using the methanol-soluble fraction from the Amberlite XAD-2 column. The compound was eluted from ITLC sheets by repeated treatment with aqueous methanol and purified by passing it through a neutral Alumina column

* Amberlite XAD-2 resin, a nonionic styrene-divinyl benzene copolymer from Rohm & Haas Company, Philadelphia, Pa.

† Uniform glass microfiber sheets impregnated with Silica gel obtained from Gelman Instrument Company, Ann Arbor, Mich.

‡ Radiochromatograms were scanned by transferring sectioned 0·5 or 1 cm planimetric strips of glass fiber Silica gel-impregnated sheets to counting vials, addition of 0·5 ml water and 10 ml Bray's solution, and assay of radioactivity in a liquid scintillation counter.

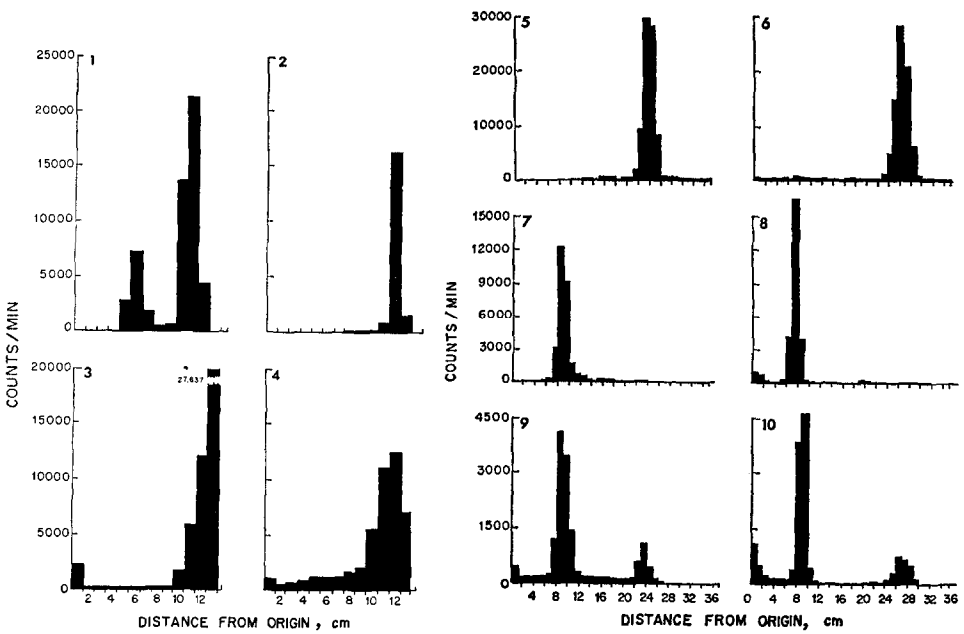

FIG. 1. Radiochromatographic scans of: (1) Methanol-soluble residue (from Amberlite XAD-2 column on which rat urinary metabolites had been adsorbed) on ITLC with *n*-butanol–acetic acid–water (35:3:10, v/v). Polar metabolites, R_f 0·44; and mixed free bases, R_f 0·80. (2) Mixed free bases from (1) (R_f 0·80) eluted from ITLC sheets (20 × 20 cm), rerun in *n*-butanol–acetic acid–water (35:3:10, v/v). (3) Mixed free bases isolated from ITLC (2) on ITLC with solvent system ethyl acetate–methanol–conc. NH_4OH (17:2:1, v/v); peaks, R_f 0·0 and R_f 0·90. (4) Compound with R_f 0·0 from (3) run on ITLC with *n*-butanol–acetic acid–water (35:3:10, v/v); peak, R_f 0·90. (5) Paper chromatography of mixed free bases separated from ITLC in (1) in solvent system, *n*-butanol–acetic acid–water (4:1:2, v/v); major peak, R_f 0·68, and minor peak, R_f 0·48. (6) Paper chromatography of mixed free bases in *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v); major peak, R_f 0·75. (7) Mixed conjugates (polar metabolites) separated from ITLC in (1) paper chromatographed in *n*-butanol–acetic acid–water (4:1:2, v/v); major peak, R_f 0·25. (8) Mixed conjugates (polar metabolites) separated from ITLC in (1) paper chromatographed in *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v); major peak, R_f 0·23, and minor, R_f 0·0 to 0·03. (9) Methanol-insoluble residue from Amberlite XAD-2 column, paper chromatographed in *n*-butanol–acetic acid–water (4:1:2, v/v). Morphine, R_f 0·65; morphine-3-glucuronide, R_f 0·24; minor, R_f 0·03. (10) Methanol-insoluble residue from Amberlite XAD-2 column, paper chromatographed in *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v). Morphine-3-glucuronide, R_f 0·28; minor, R_f 0·03; morphine, R_f 0·75.

(1 × 10 cm) and elution with a large volume of 50% aqueous methanol (400 ml). The residue from this eluate was soluble in water and in methanol, but sparingly so in ethanol. The ultraviolet spectrum in spectral grade methanol is shown in Fig. 2. This compound showed absence of a free phenolic group by Folin–Ciocalteau and ferric chloride–ferricyanide reagent.* Acid hydrolysis of this compound with 2·4 N HCl for 1 hr at 15–20 pounds of pressure, neutralization to pH 9, and subsequent extraction as described previously for morphine gave a residue which on ITLC with ethyl acetate–methanol–ammonia (17:2:1, v/v) established only a minor conversion to morphine. The major portion of radioactivity, however, was not due to morphine.

* Sequential treatment with freshly prepared alkaline potassium ferricyanide and acid ferric chloride solution; a Prussian blue coloration or a precipitate indicated presence of a phenolic group.

The compound purified on neutral Alumina was paper chromatographed in an *n*-butanol–acetic acid–water (4:1:2, v/v) system to give a peak of radioactivity at R_f 0·28 (nonradioactive morphine reference, R_f 0·61), and in an alkaline solvent system of *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v), a peak of radioactivity, R_f 0·0 to 0·06 (nonradioactive morphine reference, R_f 0·77), was obtained.

Polar metabolite from methanol-soluble fraction from Amberlite XAD-2 column. The polar metabolites consisting of mixed conjugates separated from ITLC were chromatographed on Whatman 3MM paper with solvent system *n*-butanol–acetic acid–water (4:1:2, v/v) and produced a major peak at R_f 0·25 due to morphine-3-glucuronide [Fig. 1(7)], and with solvent system *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v), a major peak at R_f 0·23 due to morphine-3-glucuronide and a minor peak at R_f 0·0 to 0·03 [Fig. 1(8)], probably identical to the minor peak observed in the acid system.

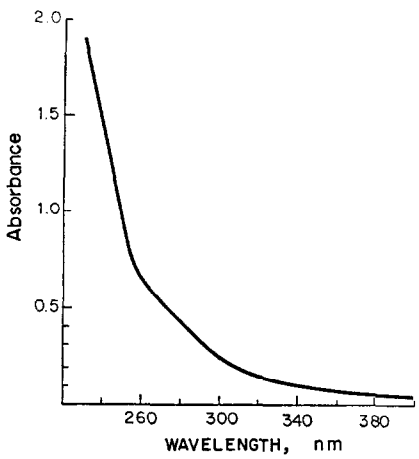

FIG. 2. Ultraviolet absorption spectrum of a new metabolite of morphine *in vivo* in spectral grade methanol.

Methanol-insoluble fraction from Amberlite XAD-2 column. The insoluble greenish brown residue, which was dissolved in water and chromatographed on Whatman 3 MM paper with *n*-butanol–acetic acid–water (4:1:2, v/v), gave three peaks of radioactivity: R_f 0·65, (contaminating) morphine; major peak, R_f 0·24; and minor peak, R_f 0·03 [Fig. 1(9)]. Paper chromatography in an alkaline system, *n*-butanol–*n*-propanol–2 N NH_4OH (2:1:1, v/v), also produced three peaks of radioactivity: major peak at R_f 0·28, minor at R_f 0·03, and another due to contaminating morphine at R_f 0·75 [Fig. 1(10)].

Metabolism of morphine in vitro *by rat brain homogenates.* Cerebral cortical hemispheres from five male rats (100–150 g) were homogenized in ice-cold Krebs–Henseleit buffer (pH 7·4) and a 10% homogenate containing morphine-*N*-methyl-^{14}C (5000 μg) was incubated with shaking in volumes of 5 ml under oxygen at 37° for 90 min. The homogenates were then pooled, supernatant was removed by centrifugation and debris was washed twice with small volumes of buffer. The pooled supernatants were filtered through a Buchner funnel and the filtrate was passed through a column of

Amberlite XAD-2 resin. After washing the resin with water, the column was eluted with 300 ml methanol containing 10 ml conc. ammonia. The residue from the organic phase obtained on evaporation *in vacuo* on thin-layer chromatography with solvent system ethyl acetate–methanol–conc. ammonia (17:2:1, v/v) and radioscanning gave two distinct peaks of radioactivity, one at the origin, R_f 0·0, and the other at the solvent front, R_f 1·0 (morphine). Chromatography on preparative ITLC sheets (20 × 20 cm) with the same solvent system gave larger quantities of compound, R_f 0·0. A similar radioscan was also obtained by thin-layer chromatography with solvent system *n*-butanol–acetic acid–water (35:3:10, v/v). The brain debris, after washing with buffer, still retained radioactivity, part of which was removed by two extractions with aqueous 0·1% Triton X-100 solution. A total conversion of 9–10 per cent of morphine to the unknown metabolite was obtained in this experiment.

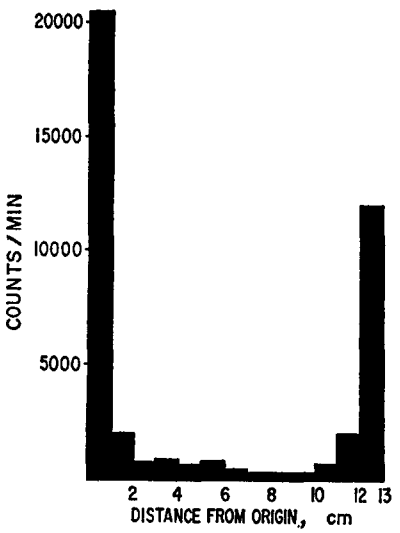

FIG. 3. Radioscan of a thin-layer chromatogram of the acid-hydrolyzed morphine metabolite (R_f 0·0) obtained *in vitro* with rat brain homogenates.

The aqueous solution of the eluted metabolite containing nonradioactive morphine carrier was autoclaved with 20% by volume of conc. hydrochloric acid for 1 hr at 15–20 pounds of pressure, neutralized with dil. NaOH to pH 7·0, and chromatographed and eluted from Amberlite XAD-2 column as described above. Thin-layer chromatography of the concentrated eluate with ethyl acetate–methanol–conc. ammonia (17:2:1, v/v) showed radioactivity at the origin and approximately 36 per cent at the solvent front (morphine, R_f 1·0) (Fig. 3). Reversible binding of morphine with brain proteins was thus partially ruled out. Tentative evidence for the presence of two vicinal phenolic groups in the new metabolite was obtained with cobalt nitrate color reagent,[20] in addition to the positive test for phenolic groups with Folin-Ciocalteau, potassium ferricyanide–ferric chloride and naphthanil diazoblue* reagents.

* Prepared fresh by dissolving 50 mg diazoblue B in 15 ml of 0·25 M borate buffer (pH 9·0) and filtered for use as spray reagent.

Minor conversion in vitro *of morphine to its* N-*oxide by rat liver homogenates.* Morphine-*N*-methyl-^{14}C (200 μg) in volumes of 2 ml, male rat liver homogenate (10% in 0·1 M phosphate buffer, pH 7·4) containing 1·5 mg NADPH was incubated for 2 hr under oxygen at 37° with shaking in a Dubnoff metabolic shaker. After incubation, the homogenates were pooled, the solution was brought to pH 9 with dil. NaOH and extracted repeatedly as previously described.[17] The organic extracts were pooled and to the aqueous phase 95% ethanol was added to precipitate the proteins; the aqueous ethanolic supernatant was centrifuged and evaporated to dryness at 45° under nitrogen. The precipitated protein was rewashed with ethanol and all ethanol extracts were combined. Residue from the aqueous ethanol extracts was repeatedly extracted with methanol and the methanol extracts were combined with the chloroform–isopropanol extract, giving approximately 80 per cent of the total radioactivity in the organic phase. This fraction on TLC with ethyl acetate–methanol–conc. ammonia (17:2:1, v/v) showed the presence of approximately 5·5 per cent of another radioactive product (R_f 0·47), besides morphine (R_f 0·90), and minor amounts of normorphine (R_f 0·73) detected with ninhydrin. TLC and paper chromatographic experiments gave suggestive evidence that this conversion product was morphine-*N*-methyl-^{14}C oxide.

The residue from the aqueous ethanol extract, insoluble in methanol, was dissolved in water, freed of salts by adsorption on Amberlite XAD-2 resin and eluted with methanol. The residue from this eluate on TLC with *n*-butanol–acetic acid–water (35:3:10, v/v) gave evidence of minor amounts (3–4 per cent) of morphine-3-glucuronide (R_f 0·30 to 0·33) and approximately 6–7 per cent of a more polar metabolite (R_f 0·07 to 0·15) which was positive to both iodoplatinate and ninhydrin.

Attempted preparation of 2-*hydroxymorphine at ambient temperature by a one-step route.* Oxidation of morphine sulfate by alkaline potassium ferricyanide to an intermediate oxidation product was done by slightly modifying the procedure of Woods *et al.*[21] Morphine sulfate (3·25 g) was dissolved in 25 ml distilled water, sodium hydroxide (1 g) was added and the solution transferred to a 250-ml separatory funnel containing 125 ml chloroform. A solution of potassium ferricyanide (2·885 g in 12·5 ml water) was added to the contents in the separatory funnel and the mixture was vigorously shaken for 5 min. The chloroform layer was transferred to a second separatory funnel and washed twice with 25-ml portions of distilled water. The chloroform extract was dried over anhydrous sodium sulfate, filtered and evaporated *in vacuo* under nitrogen to give 1·26 g of a greenish yellow glassy friable product.

This product was dissolved in a minimum volume of water followed by 50 ml of 3% hydrogen peroxide and 4 ml of 0·5% aqueous cupric sulfate. The mixture was stirred for 2·5 hr at ambient temperature, the insoluble residue (pseudomorphine) filtered by suction, and the reddish colored filtrate evaporated to dryness *in vacuo*. The residue thus obtained was dissolved in a minimum volume of water, the solution was transferred to a 40-ml centrifuge tube and methanol added to precipitate a light yellow product (435 mg). This procedure was repeated three times and the final product was obtained on drying at ambient temperature *in vacuo*. This compound was very soluble in water, sparingly so in methanol and ethanol, and insoluble in chloroform and ether. It was susceptible to heat and the aqueous solution, on keeping at ambient temperature or on warming, deposited insoluble polymeric material. It decomposed at 260–270° without melting at 300°. *Anal:* Calcd. for $C_{17}H_{19}NO_4$–5

H_2O: C, 52·17; H, 7·41; N, 3·58. Found: C, 52·15; H, 5·54; N, 3·59. TLC on glass fiber sheets with: (a) solvent system ethyl acetate–methanol–conc. ammonia (17:2:1, v/v) gave a single iodoplatinate-positive spot, R_f 0·0 (R_f morphine, morphine-*N*-oxide and pseudomorphine, 1·0, 0·50 and 0·11 respectively); (b) solvent system *n*-butanol–acetic acid–water (35:3:10, v/v), R_f 0·0 (R_f morphine and pseudomorphine, 0·83 and 0·13

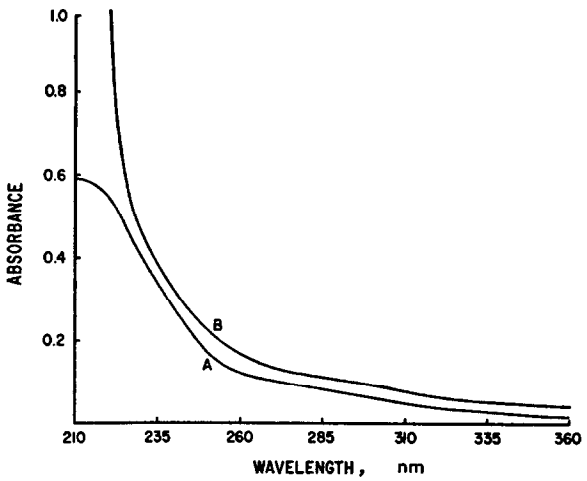

FIG. 4. Ultraviolet absorption spectrum of the compound obtained by sequential oxidation of morphine, first with alkaline ferricyanide and then with hydrogen peroxide and copper ions: (a) in 0·1 N HCl, and (b) after addition of 0·1 ml of 5 N NaOH.

respectively). Paper chromatography with: (a) solvent system *n*-butanol–acetic acid–water (4:1:2, v/v) showed a single spot, R_f 0·07 to 0·13 (R_f morphine and pseudomorphine, 0·66 and 0·40 respectively); (b) *n*-butanol–*n*-propanol–2 N ammonia (2:1:1, v/v), R_f 0·0 (R_f morphine and pseudomorphine, 0·85 and 0·81 respectively).

The ultraviolet spectrum of this compound (Fig. 4) in 0·1 N HCl (25–100 μg/ml) neither showed the maxima due to a phenolic group nor any bathochromic shift on addition of alkali. The i.r. spectrum (Fig. 5) showed a weak broad carbonyl band

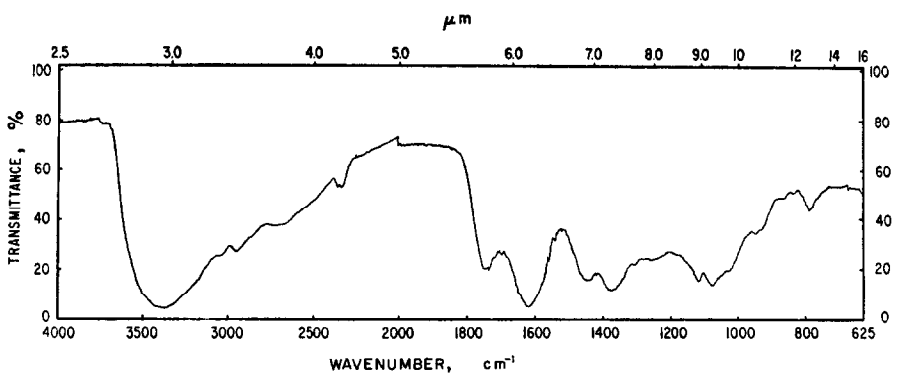

FIG. 5. Infra-red spectrum of the compound in KBr obtained by sequential oxidation of morphine, first with alkaline ferricyanide and then with hydrogen peroxide and copper ions.

(5·75 μm) but no characteristic aromatic bands. The compound exhibited only a weak fluorescence in 0·1 N HCl with emission peak at 427 nm and excitation peaks at 296 and 395 nm. A relatively weak low molecular weight mass spectrum was obtained using the direct solid probes and raising the temperature from 50 to 400°, indicating the highly polar nature of this compound. A small but distinct peak at mass 285 and a peak at mass 301 slightly above background were observed in mass spectrum. The n.m.r. spectrum in D_2O showed that the oxidation took place in the aromatic ring and not on the N–CH_3 group, or the 6-alcoholic group or 7,8 double bond. The mechanistic scheme for the formation of this oxidation product of morphine is outlined in Fig. 6, and shows the zwitterionic nature of this product.

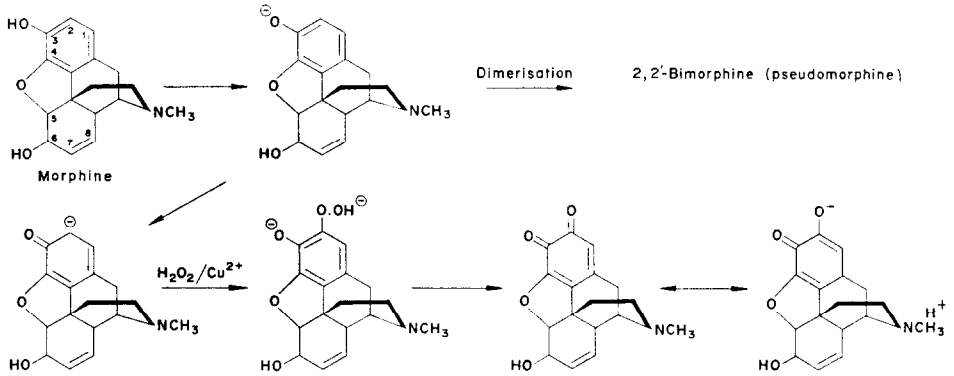

FIG. 6. Mechanistic scheme for the formation of synthetic zwitterionic 2,3-quinone from morphine by sequential oxidation, first with alkaline ferricyanide and then with hydrogen peroxide and copper ions.

The chloroform-soluble greenish yellow intermediate product obtained by alkaline ferricyanide oxidation of morphine was unstable in water at ambient temperature and within a few hours deposited a colorless precipitate of pseudomorphine characterized by its conversion to a cream colored microcrystalline hydrochloride, m.p. > 300°. *Anal:* Calcd. for pseudomorphine hydrochloride, $C_{34}H_{36}N_2O_6$–2 HCl–2 H_2O: C, 60·26; H, 6·20; N, 4·13; Cl, 10·48. Found: C, 60·63, 60·98; H, 6·41, 6·46; N, 4·13; Cl, 10·27. The identity of this compound as pseudomorphine[22] was further confirmed by u.v., i.r., fluorescence, n.m.r. spectrum (aromatic proton singlet at 1370 Hz), paper and thin-layer chromatography, and derivatization to a crystalline tetra-acetyl derivative, and comparison with an authentic sample of pseudomorphine.

RESULTS AND DISCUSSION

In 48 hr urine and feces of rats injected subcutaneously with a single 10 mg/kg dose of morphine-*N*-methyl-^{14}C, 25–30 per cent of the dose could be accounted for as free morphine and 35–40 per cent as conjugated morphine. Chromatography, enzymic hydrolysis with β-glucuronidase, and physicochemical characterization established the identity of the conjugate as morphine-3-glucuronide. *N*-dealkylation to normorphine ranged from 5 to 10 per cent of the injected dose and was easily demonstrated by the chromatographic procedures described earlier[10] and in the text.

These findings were in general agreement with results reported earlier.[11,23,24] The rest of the dose could be accounted for on the basis of a new metabolite excreted in free form (5–6 per cent of the injected dose) and partly as a conjugate which had very low R_f values in acidic and alkaline solvent systems on paper chromatography. The radioactive metabolite was soluble in water and its u.v. spectrum in dilute acid solution showed neither a distinctive maxima at 285 nm nor a bathochromic shift in alkaline solution. The possibility that this compound could be an impurity contaminated with morphine-3-glucuronide or morphine-3-ethereal sulfate, which show this maxima and no bathochromic shift, was ruled out by co-chromatography with authentic samples of these compounds. Further enzymic hydrolysis with glusulase* did not give evidence for the formation of morphine on TLC. These observations would indicate a change in the character of the aromatic ring. The absence of a free

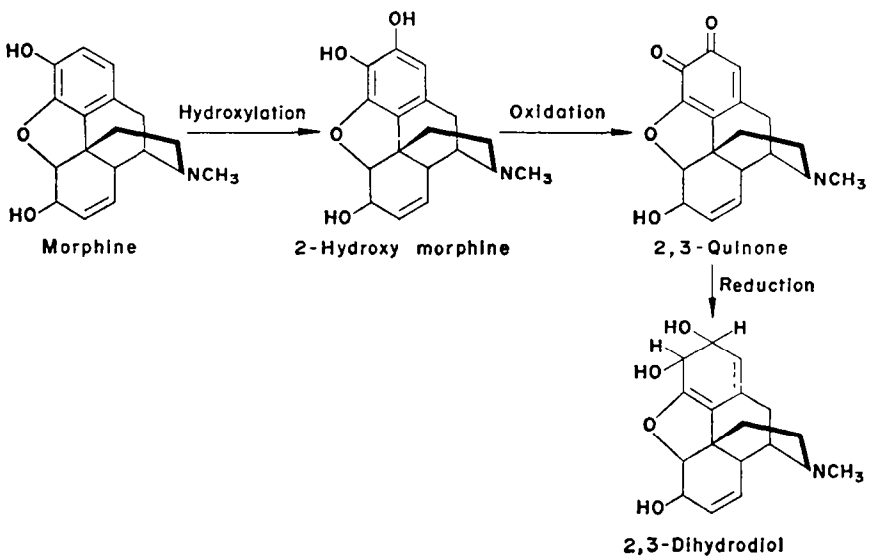

FIG. 7. Outline of the scheme for the possible formation of the 2,3-dihydrodiol urinary metabolite of morphine.

phenolic group, dissimilarity from known conjugates, e.g. 3- or 6-glucuronides or 3-ethereal sulfate, and only a minor conversion to morphine on autoclaving with 2·4 N HCl under 15–20 pounds of pressure would suggest the possible formation of either a 2,3-quinonoid or 2,3-dihydrodiol type of metabolite (Fig. 7). The urinary metabolite, however, had chromatographic characteristics different from those of a 2,3-quinonoid compound. The dihydrodiol structure has, therefore, been tentatively assigned to this metabolite. Dihydrodiols have been encountered as intermediates in enzymic hydroxylation of aromatic compounds.[25,26]

However, metabolism studies *in vitro* with rat brain homogenates furnished evidence for the conversion of morphine to a different highly polar phenolic metabolite which could not be converted back to morphine on acid hydrolysis. Its lack of similarity with known morphine metabolites and the presence of vicinal phenolic groups would suggest that this metabolite has a catechol type of structure. Metabolism studies

* A mixture of β-glucuronidase and sulfatase obtained from Endo Laboratories, Garden City, N.Y.

in vitro with rat liver homogenates also showed the formation of approximately 6–7 per cent of the catechol type of metabolite of morphine similar to that obtained with brain homogenates. In addition, small amounts of normorphine and a product (5·5 per cent) chromatographically similar to morphine *N*-oxide were also obtained. As the liver homogenates in these experiments *in vitro* were not supplemented with UDPGA, it was possible that the major metabolic pathway of morphine (3-glucuronide conjugation) *in vivo* was suppressed, leading to the formation of *N*-oxide as a minor biotransformation product by an alternative pathway.[27] Morphine *N*-oxide has not been isolated as a metabolite of morphine in normal circumstances, but only in the urine of cancer patients receiving mixtures of morphine and 2,4-diamino-5-phenylthiazole or 1,2,3,4-tetrahydro-9-amino acridine.[14]

Oxidation of morphine with alkaline ferricyanide is known to proceed via a mesomeric aryloxy free radical, subsequently leading to dimeric 2,2′-bimorphine (pseudomorphine).[22] A reinvestigation of the oxidation product[21] of morphine formed by the action of alkaline ferricyanide proved it to be a highly reactive and unstable intermediate. This could be converted to a water-soluble oxidation product (zwitterionic 2,3-quinone) in 30 per cent yield on interaction with 3% hydrogen peroxide and Cu^{2+} ions. Insoluble pseudomorphine was formed as a by-product. The chromatographic behavior of this oxidation product was similar to that of the metabolite of morphine formed *in vitro* by rat brain and liver homogenates.

Likewise, interaction of morphine with horseradish peroxidase and hydrogen peroxide at neutral pH has also been shown[28] to proceed via a free radical mechanism to yield a reactive intermediate which could also be converted to a similar oxidation product by treatment with hydrogen peroxide and copper ions. Insoluble pseudomorphine has previously been reported[29] to be formed as a by-product in this reaction. Aromatic hydroxylation of a variety of compounds catalyzed by peroxidase and hydrogen peroxide has previously been reported.[30–34]

These possible routes for one-step aromatic hydroxylation (or zwitterionic quinone formation) of morphine appear interesting in view of the fact that the microsomal NADPH-linked enzyme system, a mixed-function oxidase present in the endoplasmic reticulum of hepatic parenchymal cells, has been shown to hydroxylate *in vitro* a variety of compounds,[35–41] including morphine.[19]

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