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# Isolation and Purification of Rat Liver Morphine UDP-Glucuronosyltransferase

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**SUMMARY**

A UDP-glucuronosyltransferase (UDPGT) isoenzyme capable of morphine glucuronidation has been purified to apparent homogeneity and partially characterized from hepatic microsomes of female Wistar rats which have low 3α-hydroxysteroid UDPGT. A rapid and sensitive assay was developed to quantify morphine glucuronide formation using $^{14}$C-UDP-glucuronic acid and reverse phase C-18 minicolumns whereby radioactive glucuronides were differentially eluted from $^{14}$C-UDP-glucuronic acid. Trisacryl-DEAE and chromatofocusing chromatographic procedures were employed to separate and purify morphine UDPGT in the presence of exogenous phosphatidylcholine. The addition of phospholipid was necessary to stabilize UDPGT activities throughout the purification procedures. Morphine UDPGT was isolated to apparent homogeneity and displayed a pI of 7.9 upon chromatofocusing. A monomeric molecular weight of 56,000 was obtained. The purified enzyme reacted with morphine but not with 4-hydroxybiphenyl, *p*-nitrophenol, testosterone, androsterone, estrone, bilirubin, 4-aminobiphenyl, or α-naphthylamine. The $MgCl_2$ requirement for maximal expression of morphine glucuronidation was higher for the purified enzyme than for solubilized and intact microsomes. Codeine competitively inhibits morphine glucuronidation with an apparent $K_i$ of 1.1 mM with the purified morphine UDPGT. 4-Hydroxybiphenyl UDPGT was separated from morphine UDPGT using a chromatofocusing procedure for Emulgen 911-solubilized microsomes. An apparent pI value of 5.5 was obtained for this protein. Based on this work we conclude that morphine and 4-hydroxybiphenyl can react with separate UDPGT isoforms.

Glucuronidation is an important process in mammalian drug metabolism in which many xenobiotics and endogenous compounds are generally converted to more water-soluble metabolites. The reactions which are catalyzed by hepatic microsomal UDPGTs are bimolecular and dependent on UDPGA which supplies the glucuronic acid moiety. Substances with —OH, —$NH_2$, —COOH, and —SH groups can be glucuronidated (1) and the toxicity or pharmacologic activity of a given compound is often reduced *in vivo*; however, in certain cases, as with amine substrates, toxicity may still occur (1).

Morphine is an important drug that is metabolized primarily via glucuronidation (2). Our laboratory has studied morphine glucuronidation and the enzyme involved in catalyzing this process. We have previously separated and partially purified a UDPGT that catalyzes the glucuronidation of morphine in rat (3) and rabbit liver (4) but, because of the lability of the protein, it has resisted purification to homogeneity. However, it was possible to demonstrate that the partially purified morphine UDPGT did not catalyze *p*-nitrophenol glucuronidation (3, 4).

Bock *et al.* (5) have classified several forms of UDPGT in rat liver based largely on differential induction experiments using either 3-methylcholanthrene or phenobarbital. One form of the enzyme which was induced with 3-methylcholanthrene treatment possessed activity toward 1-naphthol and *p*-nitrophenol as well as many other substrates including morphine and 4-hydroxybiphenyl. Another form of purified transferase was induced by phenobarbital treatment (5) and was termed "GT-2." This protein was proposed to be associated with morphine, chloramphenicol, and 4-hydroxybiphenyl glucuronidation.

Our laboratory has shown that, in rat liver, *p*-nitrophenol and 1-naphthol are glucuronidated by two separate and distinct UDPGTs, one of which is inducible by 3-methylcholanthrene and the other is not inducible by pretreatment with 3-methylcholanthrene (6). Extensive characterization studies have shown that the 3-methylcholanthrene-inducible *p*-nitrophenol UDPGT has a monomeric molecular weight of 56,000 on SDS-PAGE. A 17β-hydroxysteroid UDPGT also reacts with *p*-nitrophenol and 1-naphthol but is not inducible by 3-methylcholanthrene. This protein has a monomeric molecular weight of 50,000. Thus, both forms of UDPGT would be expected to be present in liver of untreated or 3-methylcholanthrene-treated rats although they would be present at different levels.

In this study we report the purification to apparent homogeneity of morphine UDPGT and provide information on its

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**ABBREVIATIONS:** UDPGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LA, low 3α-hydroxysteroid UDPGT activity; EDTA, ethylenediaminetetraacetate.

physical and chemical characteristics. The study also gives evidence to the effect that morphine and 4-hydroxybiphenyl are glucuronidated by different proteins.

## Materials and Methods

**Chemicals.** [4-$^{14}$C]Estrone (57.0 mCi/mmol), [4-$^{14}$C]testosterone (50 mCi/mmol), [1,2-$^{3}$H]androsterone (40–60 Ci/mmol), and UDP-D-[U-$^{14}$C]-glucuronic acid (275 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [2,6-$^{14}$C]-*p*-Nitrophenol (30 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Sep-Pak C-18 minicolumns were obtained from Waters Associates (Milford, MA). Morphine sulfate was acquired from Merck and Co., Inc. (Rahway, NJ). Dithiothreitol, 4-hydroxybiphenyl, L-α-phosphatidylcholine (egg yolk, type XI-E), *p*-nitrophenol, androsterone, testosterone, α-naphthylamine, 4-aminobiphenyl, bilirubin, and morphine glucuronide were obtained from Sigma Chemical Co. (St. Louis, MO). Codeine was purchased from Mallinckrodt Chemical Works (Paris, KY). Emulgen 911 was a gift from Kao Atlas, Ltd. (Tokyo, Japan). Trisacryl-DEAE was purchased from LKB-Produkter AB (Bromma, Sweden). Reagents for the protein assay and for silver staining were obtained from Bio-Rad (Richmond, CA). Chromatofocusing materials were purchased from Pharmacia, Inc. (Piscataway, NJ). All other chemicals were of the best quality available.

**Animals.** Female Wistar rats (200–250 g) were acquired from Charles River Breeding Laboratories (Wilmington, MA). All animals were treated with phenobarbital intraperitoneally (80 mg/kg), each day for 4 days. Rats were sacrificed 24 hr after the last dose. A sample of the microsomes prepared from each rat was analyzed for 3α-hydroxysteroid (androsterone) UDPGT activity in order to determine which rats were LA rats. Livers from LA rats were used in order to provide microsomal preparations with low levels of this enzyme. This was done to markedly reduce contamination of preparations of purified morphine UDPGT with 3α-hydroxysteroid UDPGT.

**Assays.** Morphine glucuronide formation was measured using a modification of the assays reported by Kirkpatrick *et al.* (7) and Rane *et al.* (8). The final volume of the reaction mixture was 0.14 ml and it was composed of 50 mM Tris-acetate, pH 8.4; 10 mM magnesium chloride; 0.5 mg of phosphatidylcholine/ml; 5 mM morphine base, supplied as morphine sulfate; and 5 mM $^{14}$C-UDPGA with a specific activity of approximately 40 nCi/reaction mixture. An amount of enzyme was used that yielded linear product formation with respect to both protein concentration and time. The reactions were started with the addition of $^{14}$C-UDPGA and the samples were then incubated for 20 min at 37°. The reactions were stopped by the addition of 7 ml of ice-cold 1 M ammonium acetate, pH 9.2. The complete volume was then loaded onto a reverse phase C-18 minicolumn (Sep-Pak, Waters Associates) and the matrix was washed with 10 ml of 10 mM ammonium acetate, pH 9.2. At this point, the radiolabeled morphine glucuronide remained absorbed in the matrix while the radioactive UDPGA eluted in the void volume. The morphine glucuronide was then eluted from the column with 5 ml of absolute methanol and it was quantified by scintillation counting techniques. The product, morphine glucuronide, was verified on high performance liquid chromatography and recovery averaged 95%.

4-Hydroxybiphenyl glucuronidation was determined using the same method as that for morphine glucuronidation except for the following modifications: a final reaction mixture of 0.2 ml was used and the concentration of 4-hydroxybiphenyl was 0.5 mM. The rate of product formation was linear with respect to both protein concentration and time. The reaction was started with the addition of $^{14}$C-UDPGA and the reaction mixture was incubated for 10 min. The rest of the procedure was performed as described for morphine glucuronidation. Testosterone, androsterone, estrone, and *p*-nitrophenol glucuronidation activities were assayed according to the methods reported by Falany and Tephly (6). Bilirubin UDPGT activity was assayed according to the method of Vermeir *et al.* (9), and the bilirubin glucuronide was identified by high performance liquid chromatography according to the method of Spivak and Carey (10). α-Naphthylamine and 4-aminobiphenyl UDPGT activities were assayed according to the method of Lilienblum and Bock (11).

Protein estimation was accomplished using the Bio-Rad (12) method with bovine serum albumin as the protein standard.

**Preparation of microsomes.** Rats were stunned and decapitated and the livers were perfused *in situ* with ice-cold 1.15% KCl. All procedures performed were done at 4° unless otherwise noted. The livers were finely minced and homogenized in 1.15% KCl with a rotor-driven Potter-Elvehjem homogenizer to achieve a 25% (w/v) homogenate. The homogenate was centrifuged at 10,000 × *g* for 15 min. The supernatant was filtered through cheesecloth to eliminate most of the fatty material. The resulting supernatant was centrifuged at 105,000 × *g* for 120 min. The microsomal pellet thus formed was redispersed in the 1.15% KCl solution and centrifuged at 105,000 × *g* for 120 min. The supernatant was removed and the pellet was overlayed with the 1.15% KCl solution and stored at −60° until used.

**Equilibration buffer.** Throughout the chromatographic procedures a standard buffer system was utilized. This equilibration buffer was composed of 25 mM Tris-acetate, 1 mM dithiothreitol, 20% glycerol, and 0.05% Emulgen 911. The pH of the buffer was varied depending on the chromatographic procedure.

**Solubilization of microsomes.** The microsomal pellets were thawed to 4° and the KCl overlay was removed. Approximately 100-125 mg of microsomal protein was dispersed per 10 ml of equilibration buffer and the pH of the buffer was adjusted depending on the procedure. The microsomes were solubilized by slowly adding Emulgen 911 until the final concentration was 1 mg Emulgen 911 per mg of protein. The microsomal suspension was stirred for 30 minutes and was centrifuged at 105,000 × *g* for 60 minutes. About 85–95% of the protein was recovered in the supernatant fraction.

**Separation and purification of morphine UDPGT.** A 70-ml column (1.6 × 35 cm) was packed with degassed anion-exchange matrix (trisacryl-DEAE) and equilibrated with approximately 30-column volumes of equilibration buffer, pH 9.1, containing 175 μg of phosphatidylcholine/ml. Once the column was washed, the solubilized microsomal preparation (pH 9.1) was added to the column. Approximately 165 mg of microsomal protein in 20 ml were put on the column. Prior to starting the linear NaCl gradient, the column was washed with 1 column volume of equilibration buffer, pH 9.1, containing 175 μg of phosphatidylcholine/ml. A linear 0–100 mM NaCl gradient was generated in a volume equal to 11 column volumes (800 ml). A single peak of morphine glucuronidation activity eluted between 55 and 75 mM NaCl. Peak fractions of morphine UDPGT were pooled and dialyzed against three changes of 20 volumes of equilibration buffer, pH 8.9, for 48 hr.

A 30-ml chromatofocusing column (0.9 × 40 cm) was packed with degassed PBE-94 ion-exchange resin and equilibrated with 50 volumes of equilibration buffer, pH 8.9, containing 175 μg of phosphatidylcholine/ml. Approximately 4.1 mg of protein from the previous column were applied. A pH 8.9–5.5 gradient was generated with the following buffer: 7% Polybuffer-74, 3% Polybuffer-96, 1 mM dithiothreitol, 20% glycerol, 0.05% Emulgen 911, and 175 μg of phosphatidylcholine/ml, and the pH was adjusted to 5.5 with 30% acetic acid. The pH gradient was generated with 10-column volumes of the buffer system and 7-ml fractions were collected. Morphine UDPGT eluted as a relatively broad peak within a pH range of 8.0–7.3. Fractions of morphine UDPGT were pooled and extensively dialyzed against three changes of 20 volumes of equilibration buffer, pH 8.9, for 48 hr.

A second column was prepared for chromatofocusing. A 20-ml column (1 × 20 cm) was packed with degassed ion-exchange resin (PBE-94) and equilibrated with 50 volumes of equilibration buffer, pH 8.9, containing 175 μg of phosphatidylcholine/ml. When the column was equilibrated the dialyzed pool of morphine UDPGT containing approximately 2.2 mg of protein was loaded. A pH 8.9–5.5 gradient was generated with approximately 20-column volumes of the following

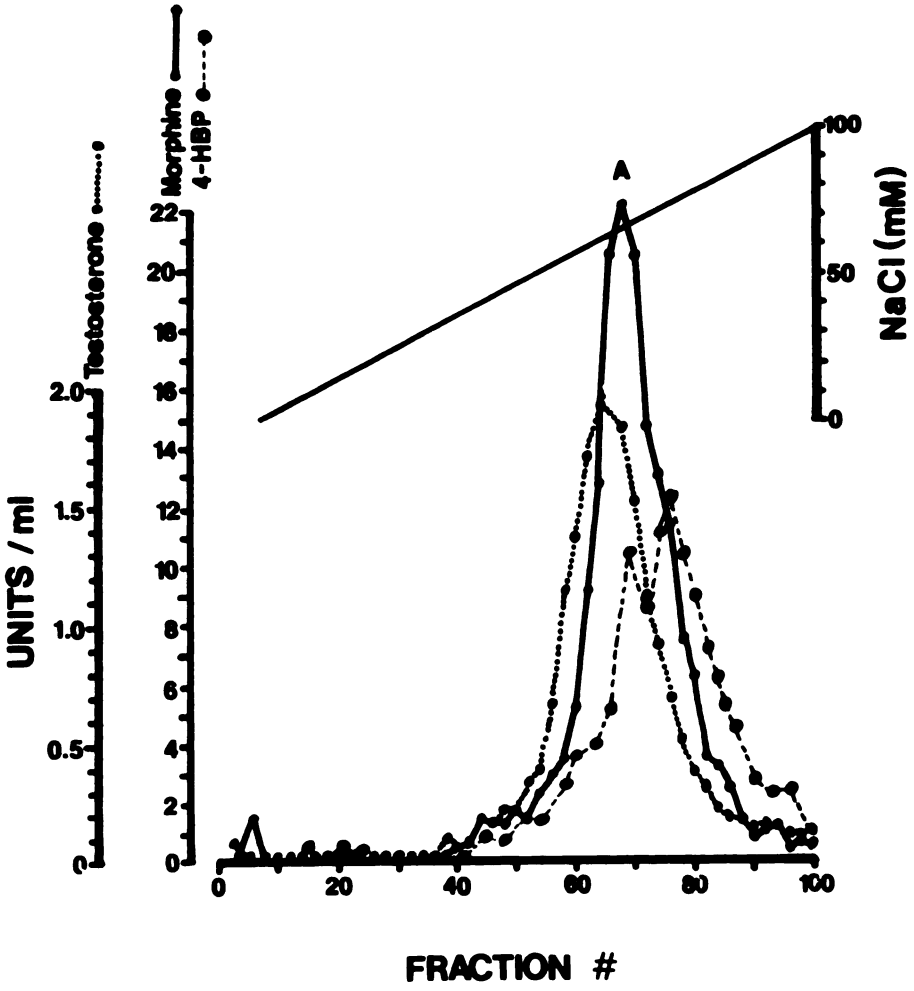


**Fig. 1.** Trisacryl-DEAE column chromatography of liver microsomes from female Wistar rats that have low 3a-hydroxysteroid UDPGT (LA rats). Solubilized microsomal protein (165 mg) was applied to a 1.6 × 35 cm Trisacryl-DEAE column equilibrated at pH 9.1. The column was then eluted with a linear 0–100 mM NaCl gradient. *A*, those fractions that were pooled (64–72) for further purification of morphine UDPGT. *4-HBP*, 4-hydroxybiphenyl.

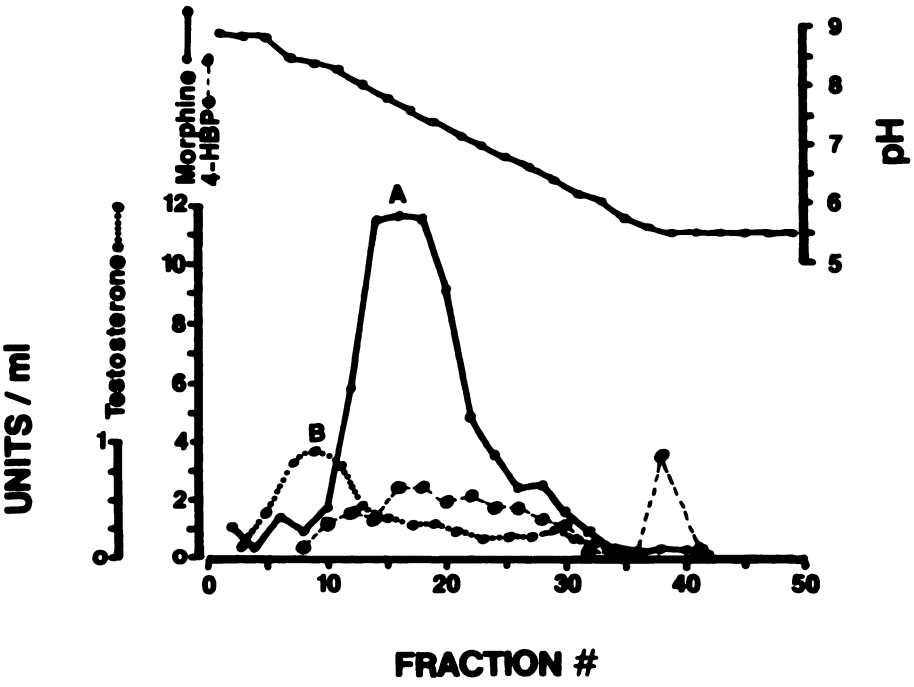


**Fig. 2.** Chromatofocusing of dialyzed pooled fractions from a trisacryl-DEAE column containing morphine UDPGT. Approximately 4.1 mg of protein were applied to the chromatofocusing column (0.9–40 cm) equilibrated at pH 8.9, and a pH 8.9–5.5 gradient was generated. *A*, those fractions that were pooled (13–21) for further purification of morphine UDPGT. *B*, those fractions containing 17$\beta$-hydroxysteroid UDPGT eluting at or about pH 8.4. *4-HBP*, 4-hydroxybiphenyl.

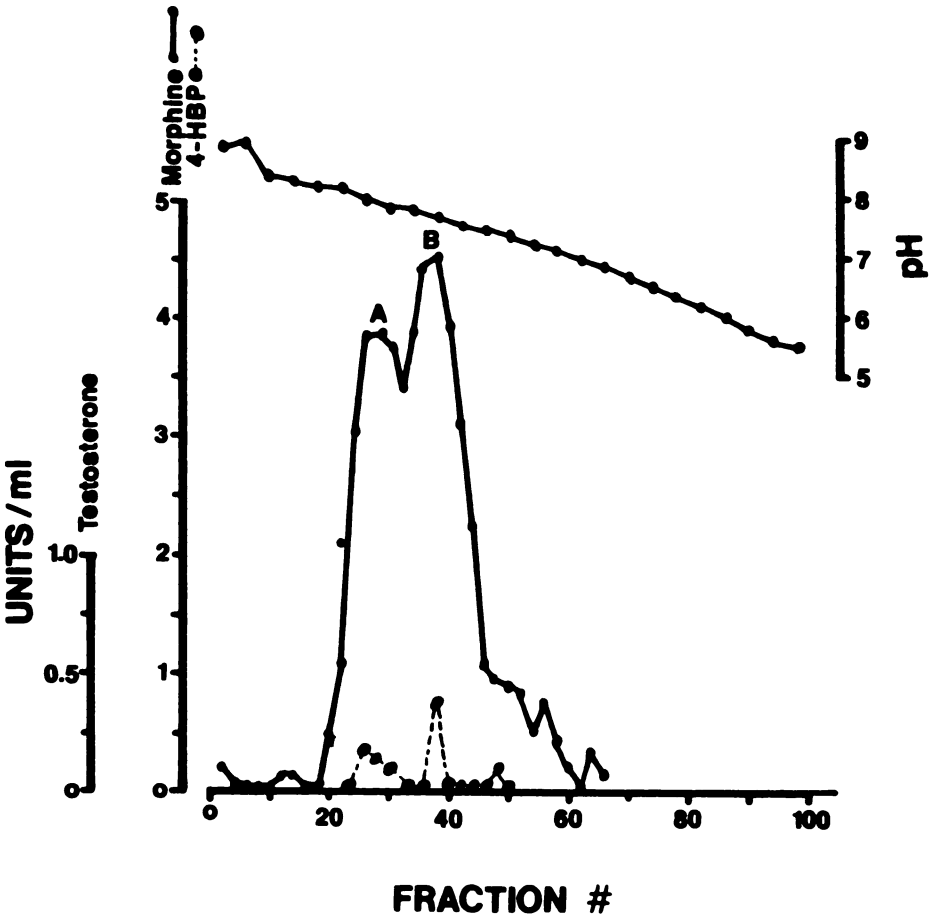


**Fig. 3.** Rechromatofocusing of dialyzed pooled fractions containing morphine UDPGT. Approximately 2.2 mg of protein were applied to a chromatofocusing column (1 × 20 cm) equilibrated at pH 8.9, and a pH 8.9–5.5 gradient was generated. *A* and *B*, two peaks of morphine UDPGT eluting at pH 7.9 and 7.7, respectively. *4-HBP*, 4-hydroxybiphenyl. No measurable activity was present toward testosterone or androsterone.

TABLE 1

**Substrate specificity for rat liver microsomal morphine UDPGT**

The limits of detection for the substrates expressed in units/ml of enzyme source are: morphine, 1.0 unit/ml; 4-hydroxybiphenyl, 0.5 unit/ml; *p*-nitrophenol, 0.21 unit/ml; androsterone, 0.02 unit/ml; testosterone, 0.05 unit/ml; estrone, 0.1 unit/ml, bilirubin, 1.0 unit/ml; 4-aminobiphenyl, 0.02 unit/ml; α-naphthylamine, 0.05 unit/ml.

| Substrate | Specific activity[a] |
|---|---|
| | *units/mg protein* |
| Morphine | 2878 |
| 4-Hydroxybiphenyl | ND[b] |
| *p*-Nitrophenol | ND |
| Testosterone | ND |
| Androsterone | ND |
| Estrone | ND |
| Bilirubin | ND |
| 4-Aminobiphenyl | ND |
| α-Naphthylamine | ND |

[a] One unit of activity represents 1 nmol of substrate conjugated/min.
[b] ND represents no detectable activity.

buffer system: 3.5% Polybuffer-74, 1.5% Polybuffer-96, 1 mM dithiothreitol, 20% glycerol, 0.05% Emulgen 911, 100 μg of phosphatidylcholine/ml, pH to 5.5 with 30% acetic acid. This pH gradient yielded two closely eluting peaks of morphine UDPGT. In some experiments, fractions from the first peak (at pH 7.9) were pooled as were fractions from a second peak (at pH 7.7).

**Separation of morphine UDPGT from 4-hydroxybiphenyl UDPGT.** Approximately 100 mg of hepatic microsomal protein from LA Wistar rats were solubilized with Emulgen 911 as described above. The solubilized microsomes were diluted five times with equilibration buffer, pH 8.6, containing 175 μg of phosphatidylcholine/ml and stored at 4° for 24 hr.

A 30-ml chromatofocusing column (0.9 × 40 cm) was packed with degassed PBE-94 ion-exchange resin and equilibrated with equilibration buffer, pH 8.6, containing 100 μg of phosphatidylcholine/ml. The solubilized microsomes were loaded onto the column and the column was washed with 2 column volumes of the buffer with which it was equilibrated. A pH gradient of 8.6–5.0 was generated with about 12 column volumes of the following buffer system: 7% Polybuffer-74, 3% Polybuffer-96, 1 mM dithiothreitol, 20% glycerol, 0.05% Emulgen 911, 100 μg of phosphatidylcholine/ml, and the pH was adjusted to 5.0 with 30% acetic acid. Morphine UDPGT eluted as a single peak at pH 7.7. 4-Hydroxybiphenyl UDPGT eluted in two peaks. The first peak was contaminated with morphine UDPGT, whereas the other peak eluted at pH 5.6 and did not possess any catalytic activity toward morphine.

**Polyacrylamide gel electrophoresis.** PAGE was performed in the presence of SDS in a Hoefer Scientific model 600 vertical slab gel unit with an ISCO model 494 electrophoresis power supply as previously outlined by Laemmli (13). Aliquots of the samples were treated with 2-mercaptoethanol and SDS to final concentratins of 10 and 2%, respectively, and placed in a boiling water bath for 10 min. Commercial molecular weight standards (Pharmacia) were treated in the same manner and used to calculate the minimum molecular weights of the samples. The polypeptide bands were visualized using the Bio-Rad silver stain reagents according to the method of Merril *et al.* (14).

## Results

**Isolation and purification of morphine UDP-GT.** Female Wistar LA rats pretreated with phenobarbital were used. LA Wistar rat liver microsomes exhibit 10% of 3α-hydroxysteroid UDPGT compared to that of high androsterone Wistar or Sprague-Dawley rats (15). We observed in preliminary experiments that this particular UDPGT isoenzyme was the major protein coeluting with morphine UDPGT; therefore, we used

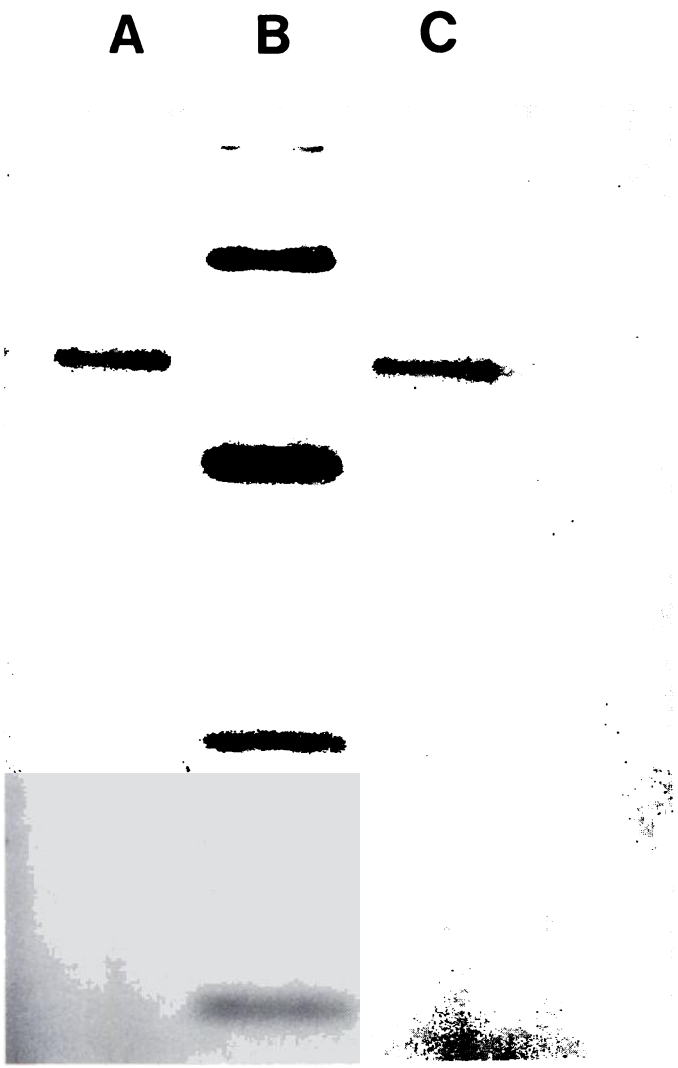


**Fig. 4.** SDS-PAGE of morphine UDPGT and commercial standards. SDS-gel electrophoresis was performed by the method of Laemmli (13). A contains pooled fractions from peak A from Fig. 3 (1 $\mu$g); B contains commercial standards (Pharmacia) with minimum molecular weights of 94,000, 67,000, 43,000, 30,000, and 20,100; and C contains pooled fractions from peak B from Fig. 3 (1 $\mu$g). Migration is from top to bottom.

microsomes from LA Wistar rats in subsequent experiments in order to minimize 3$\alpha$-hydroxysteroid UDPGT contamination. Trisacryl-DEAE ion exchange chromatography of solubilized microsomes was carried out (Fig. 1). The UDPGT activities toward testosterone and 4-hydroxybiphenyl eluted from the column in close association with the morphine UDPGT activity but not with identical elution profiles.

Fractions containing morphine UDPGT were pooled (63–72) and dialyzed, and the morphine UDPGT pool was loaded onto a chromatofocusing column and a pH gradient of 8.9–5.5 was used to resolve the morphine UDPGT (Fig. 2). Morphine UDPGT eluted from the column as a broad peak within a pH range of 8.0–7.3. 17$\beta$-Hydroxysteroid UDPGT eluted from the column with an apparent pI value of 8.4, and 4-hydroxybiphenyl UDPGT did not elute from the column with a specific association to pH. This step separated morphine UDPGT from 17$\beta$-hydroxysteroid UDPGT and from 4-hydroxybiphenyl UDPGT to some extent. The fractions containing peak activities of morphine UDPGT were pooled (13–21) and dialyzed.

A second chromatofocusing column was used to purify the morphine UDPGT present in the pooled chromatofocusing fractions with a pH 8.9–5.5 gradient (Fig. 3). Morphine UDPGT was eluted as two apparent consecutive peaks of activity at pH 7.9 and 7.7, respectively. No detectable activity for any steroid UDPGT was found throughout the morphine UDPGT peaks, and 4-hydroxybiphenyl UDPGT was either absent or at the limits of detectability (0.5 unit/ml). The apparent pattern of resolution of UDPGTs where two peaks of activity are seen after chromatofocusing has been noted in purification procedures for other UDPGTs (6).

The pooled fractions containing morphine UDPGT were studied further. No detectable activity was observed toward *p*-nitrophenol, estrone, androsterone, testosterone, 4-hydroxybiphenyl, $\alpha$-naphthylamine, 4-aminobiphenyl, or bilirubin (Table 1).

The pooled fractions of morphine UDPGT (pool A, Fig. 3) were analyzed by SDS-PAGE (Fig. 4) and stained by the silver method (14). A single polypeptide band was observed with an apparent subunit molecular weight of 56,000. This subunit molecular weight agrees with that reported by Bock *et al.* (5) for the isoform GT-2. Nonetheless, our preparation was not capable of glucuronidating 4-hydroxybiphenyl. Furthermore, it has been possible to physically separate morphine and 4-hydroxybiphenyl UDPGT activities using a chromatofocusing procedure and Emulogen 911-solubilized liver microsomes.

A summary of a representative purification procedure for morphine UDPGT to homogeneity is shown in Table 2. The specific activity for homogeneous morphine UDPGT is approximately 169 times that of liver microsomes. All of the studies presented concerning the purified morphine UDPGT were carried out in the pooled fractions of peak A from Fig. 3. The pooled fractions from peak B from the same figure displayed a substrate specificity identical to that of pool A. However, after SDS-PAGE, minor contaminating polypeptides were usually visualized after silver stain (Fig. 4). We believe that morphine UDPGT in pools A and B from Fig. 3 are the same protein.

TABLE 2

**Summary of the purification procedures for rat liver microsomal morphine UDPGT**

| | Total protein | Specific activity | Total activity[a] | Yield | Purification factor | $K_m$ morphine | $K_m$ UDPGA |
|---|---|---|---|---|---|---|---|
| | *mg* | *units/mg* | *units* | % | | *mM* | |
| Native microsomes | 194 | 17 | 3298 | 100 | 1 | 0.2 | 0.7 |
| Solubilized microsomes | 165 | 43 | 7095 | 215 | 2.5 | 2.7 | |
| Trisacryl-DEAE | 4.1 | 380 | 1555 | 47 | 22.4 | 1.8 | |
| Chromatofocusing I | 2.2 | 1143 | 517 | 15.7 | 67.2 | 3.1 | |
| Chromatofocusing II | 0.075 | 2878 | 215 | 6.5 | 169.3 | 7.4 | 0.08 |

[a] One unit of activity represents 1 nmol of substrate conjugated/min.

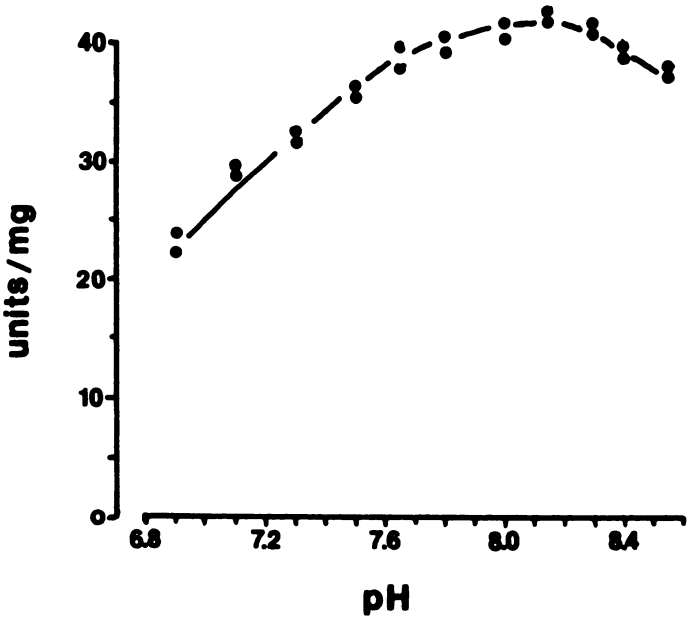


Fig. 5. Effect of pH on morphine UDPGT from Emulgen 911-solubilized rat liver microsomes.

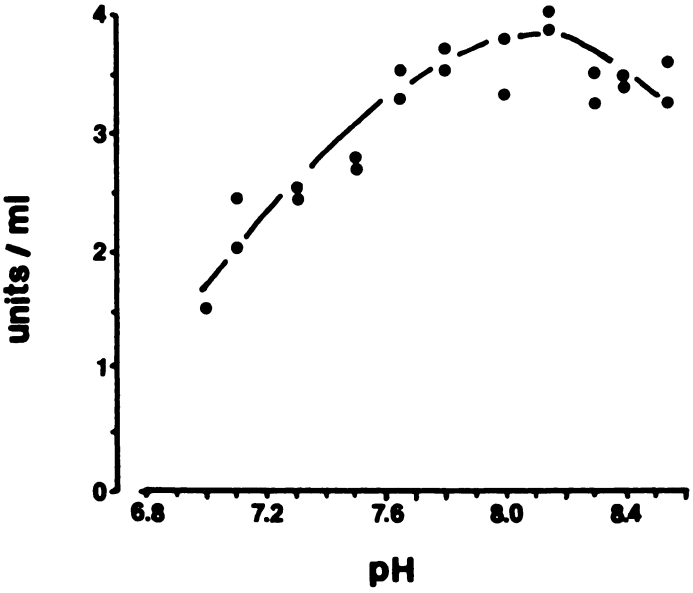


Fig. 6. Effect of pH on purified morphine UDPGT from rat liver microsomes.

The effect of pH on morphine UDPGT from solubilized liver microsomes and the purified enzyme preparation is shown in Figs. 5 and 6, respectively. The pH effect on morphine glucuronidation is similar in both preparations, suggesting that the purification of the enzyme does not alter its pH requirements for the reaction which remains at an optimum of about 8.1. It also argues that the purified morphine UDPGT corresponds to that activity measured in solubilized liver microsomes and that the presence of exogenous phosphatidylcholine present throughout the purification does not alter its pH requirement.

The initial rates of morphine conjugation as a function of $MgCl_2$ concentration in the presence of 1 mM EDTA was measured in native and solubilized microsomes as well as in the purified morphine UDPGT preparation. It was observed that as one proceeds in the purification of this enzyme activity

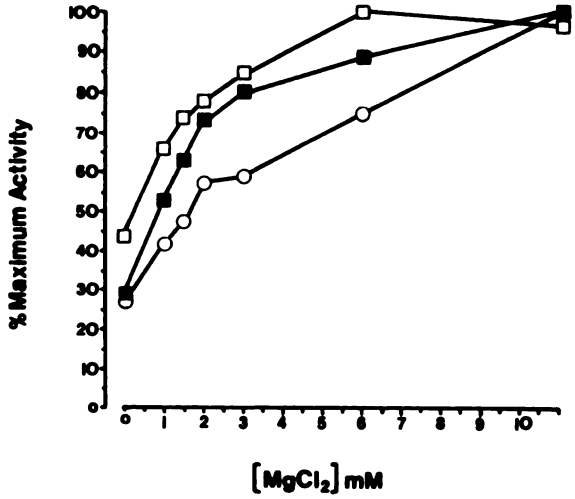


Fig. 7. Effect of $MgCl_2$ on morphine UDPGT from native microsomes (□), Emulgen 911-solubilized microsomes (■), and purified morphine UDPGT (○) in the presence of 1 mM EDTA.

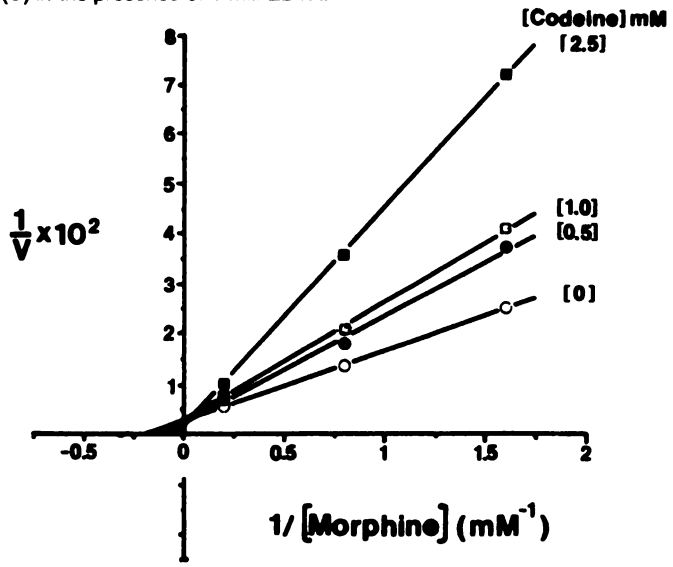


Fig. 8. Inhibition of purified morphine UDPGT by codeine. The concentration of morphine was varied (0.625–5 mM) in the presence of 5 mM UDPGA. A double reciprocal plot of initial velocity versus the concentration of morphine is presented. Velocity is expressed as nmol of morphine conjugated/min/mg of protein. Each *point* represents the mean of three determinations.

from native to solubilized microsomes to the purified preparation, there is a need for higher $MgCl_2$ concentrations in order to reach maximal rates of morphine glucuronidation under the conditions studied (Fig. 7).

Kinetic studies were done to determine the apparent Michaelis constants for morphine and UDPGA in native and solubilized microsomes as well as in the purified preparation (Table 2). The apparent $K_m$ for morphine in native liver microsomes was determined to be 0.2 mM, a value that agrees with that reported by Sanchez *et al.* (3). Once the microsomes were solubilized with Emulgen 911, the apparent $K_m$ for morphine increased to 2.7 mM, indicating a decreased affinity of the enzyme for this substrate with the addition of detergent. Upon further purification, the apparent $K_m$ for morphine increases until it reaches 7.4 mM in the apparently homogeneous morphine UDPGT preparation. In contrast, it was observed that the apparent $K_m$ for UDPGA of 0.7 mM in the native liver

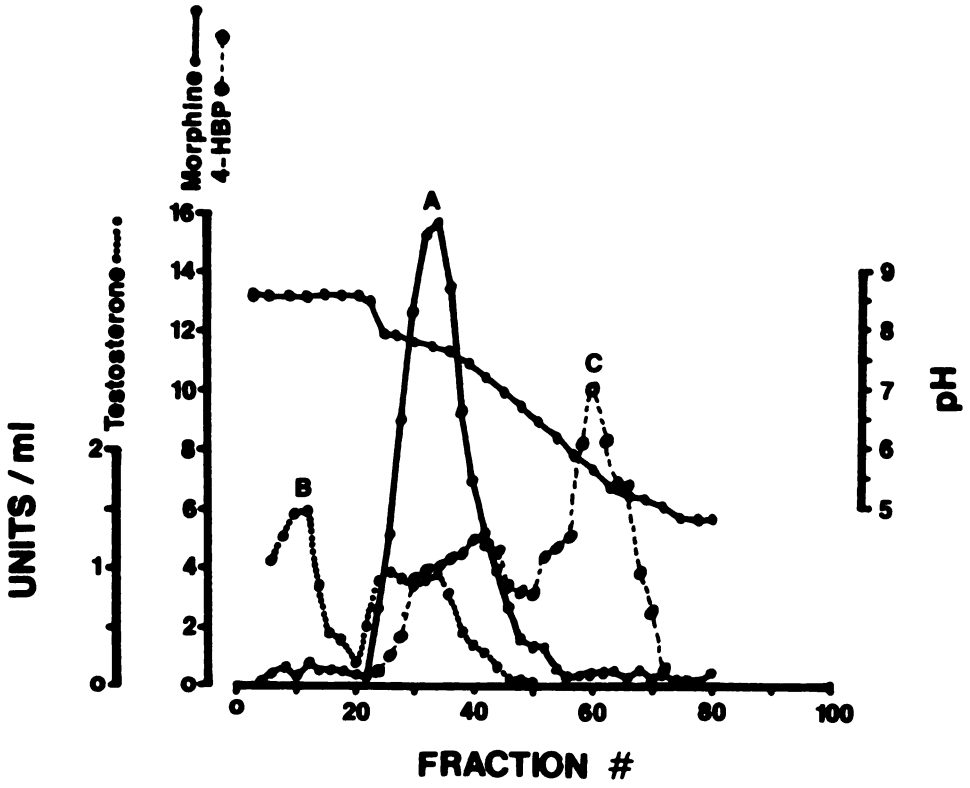


**Fig. 9.** Chromatofocusing of liver microsomes from female LA Wistar rats. One hundred mg of solubilized microsomal protein were applied to a chromatofocusing column (0.9–40 cm) equilibrated at pH 8.6. The column was eluted with a pH 8.6–5.0 gradient. *A*, those fractions containing morphine UDPGT contaminated with 17 $\beta$-hydroxysteroid UDPGT and 4-Hydroxybiphenyl UDPGT and eluting at pH 7.7. *B*, those fractions containing peak activity of 17 $\beta$-hydroxysteroid UDPGT eluting at pH 8.4. *C*, those fractions containing 4-hydroxybiphenyl UDPGT eluting at pH 5.6.

microsomes was decreased to 0.08 mM with the purified morphine UDPGT.

Sanchez *et al.* (4) demonstrated that a series of opiate agonists and antagonists was capable of inhibiting morphine glucuronidation. Codeine is a narcotic agonist and a structural analogue of morphine, differing from it only in that the phenolic group at the 3-position is methylated. Inhibition of morphine glucuronidation in the purified preparation by codeine was seen and it was competitive with morphine, with an apparent $K_i$ of 1.1 mM (Fig. 8).

**Separation of morphine UDPGT from 4-hydroxybiphenyl UDPGT.** It was possible to rapidly separate morphine UDPGT from 4-hydroxybiphenyl UDPGT by another procedure. Female LA Wistar rats were treated with phenobarbital as described in Materials and Methods. Approximately 100 mg of microsomal protein were solubilized with Emulgen 911 and loaded onto a chromatofocusing column preequilibrated at pH 8.6 with equilibration buffer containing 100 $\mu$g of phosphatidylcholine/ml. An elution profile was obtained as seen in Fig. 9. Morphine UDPGT eluted from the column at about pH 7.7. 4-Hydroxybiphenyl UDPGT eluted in two peaks. The first one eluted with morphine UDPGT and a second one, devoid of morphine UDPGT activity, eluted at pH 5.6. This result shows that a UDPGT isoenzyme catalyzing 4-hydroxybiphenyl glucuronidation can be isolated free of activity toward morphine. As seen in previous results, a morphine UDPGT was isolated with no activity toward 4-hydroxybiphenyl. This work does not rule out the possibility that another UDPGT may react with both morphine and 4-hydroxybiphenyl and is lost over the course of purification.

## Discussion

We have known for some time that morphine UDPGT was very labile, especially after microsomes were solubilized and subjected to chromatographic separatory procedures. We have also known that the morphine UDPGT could not be reconstituted by addition of exogenous phospholipid once activity was lost. However, in the current study we have shown that, when exogenous phosphatidylcholine was added to the chromatography buffers, both the recovery and the stability of morphine UDPGT were markedly improved. This finding has allowed us to proceed through a number of purification procedures and to purify morphine UDPGT to apparent homogeneity. The substrate specificity of the morphine UDPGT is quite narrow. It is capable of reacting with morphine and is competitively inhibited by codeine. However, no reaction was observed with *p*-nitrophenol, testosterone, androsterone, estrone, $\alpha$-naphthylamine, 4-aminobiphenyl, bilirubin, or 4-hydroxybiphenyl. Both Bock *et al.* (5) and Burchell (16) have reported on the purification of morphine UDPGT and considered that morphine glucuronidation was associated with more than one activity. We have not found morphine glucuronidation associated with *p*-nitrophenol, 3$\alpha$-hydroxysteroid, or 17$\beta$-hydroxysteroid UDPGTs, and we have observed only one protein in rat hepatic microsomes to be capable of catalyzing morphine glucuronidation. This does not rule out the possibility that this UDPGT isoenzyme may conjugate some, as yet unknown, endogenous substrates. It is also probable that other exogenous substrates will be identified for this enzyme.

Bock *et al.* (5) have reported on the isolation of a UDPGT from hepatic microsomes of phenobarbital-treated rats (termed "GT-2"). This protein appeared to catalyze the glucuronidation of both morphine and 4-hydroxybiphenyl. We have been able to isolate one enzyme from hepatic microsomes of phenobarbital-treated rats which catalyzes morphine glucuronidation but does not react with 4-hydroxybiphenyl. Also, we have now been able to separate a 4-hydroxybiphenyl UDPGT which does

not react with morphine. Therefore, "GT-2" is apparently not a single protein.

It is interesting to note that the monomeric molecular weight of morphine UDPGT is about 56,000. This molecular weight is essentially identical to that reported for the *p*-nitrophenol UDPGT obtained from livers of 3-methylcholanthrene-treated rats (6). The fact that two different UDPGTs with similar molecular weights are present in the same liver highlights the problem faced by employing only SDS-PAGE as a measure of homogeneity. Tukey and Tephly (17) observed two isoenzymes of UDPGT from rabbit liver microsomes with identical monomeric molecular weights. In those studies and in the current study other parameters were used in order to demonstrate qualitative differences in UDPGTs. These included pI values, substrate specificity, differential inducibility, and the use of selected inhibitors.

Detergent addition to the liver microsomes in order to solubilize morphine UDPGT leads to alterations in the apparent kinetic constants of the enzyme for morphine and UDPGA. The apparent $K_m$ for morphine in the native microsomes (no detergent added) is 0.02 mM, which increases to 2.7 mM after solubilization of the microsomes, representing a 14-fold increase in apparent $K_m$ for this substrate. Further purification of morphine UDPGT increases the apparent $K_m$ for morphine to 7.4 mM in the homogeneous preparation. It is clear that the initial addition of detergent to solubilize the microsomes has the greatest impact on the apparent $K_m$ value of morphine UDPGT for morphine. In contrast, purification of the enzyme reduces its apparent $K_m$ for UDPGA by approximately 10-fold as compared with that observed in native microsomes. It could be argued that, upon solubilization of morphine UDPGT by Emulgen 911, the enzyme is transferred from a hydrophobic environment in which morphine preferentially partitions as compared with UDPGA to a less hydrophobic environment where morphine does not dissolve as well and where UDPGA, due to its increased solubility, has better access to the enzyme. It can also be argued that extraction of morphine UDPGT from its native membrane environment by Emulgen 911 imposes changes in the enzyme's conformation which result in the observed alterations in the kinetic parameters. Probably a combination of these and other complex events is responsible for the changes seen in the enzymatic properties of morphine UDPGT as it is purified.

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