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Morphine Metabolism

IV. Studies on the Mechanism of Morphine:Uridine Diphosphoglucuronyltransferase and Its Activation by Bilirubin

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SUMMARY

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Previous studies have shown the activation of morphine glucuronidation in hepatic microsomes by bilirubin. This report shows that morphine glucuronide synthesis through microsomal UDP-glucuronyltransferase (EC 2.4.1.17) proceeds via a sequential ordered mechanism in the absence and presence of bilirubin and Triton X-100. Bilirubin markedly increases the V_{max}, slightly increases the K_a for morphine, and has no effect on the K_a for UDP-glucuronic acid. The activation occurs in intact microsomes but not with a solubilized enzyme preparation. These results may have significance with respect to the stimulation of morphine glucuronidation *in vivo*.

INTRODUCTION

Previous studies from this laboratory showed that bilirubin markedly activates the glucuronidation of morphine and *p*-nitrophenol in rat liver microsomes and increases the rate of morphine glucuronide and *p*-nitrophenylglucuronide excretion in the urine (1, 2). These studies suggest that bilirubin may play a role as an activator of UDP-glucuronyltransferase(s) *in vivo* as well as *in vitro*. Although the mechanism of bilirubin activation has not been studied extensively, preliminary data suggested that it may act like a detergent (2). Detergent activation apparently results in the modification of the phospholipid environment associated with the enzyme in the microsomal membrane (3–5).

The purpose of this study was to investigate the nature of the enzymatic mechanism involved in the formation of morphine glucuronide in native microsomes and to study the influence of bilirubin and a detergent, Triton X-100.

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MATERIALS AND METHODS

Bilirubin, UDP-glucuronic acid, and Triton X-100 were purchased from Sigma Chemical Company. Morphine sulfate was obtained from Mallinckrodt Laboratories; [*N-methyl*-^{14}C]morphine hydrochloride (54 mCi/mmole) was obtained from Amersham/Searle. Other reagents used were of the highest purity available.

Sprague-Dawley male rats weighing 200–300 g (Simonsen Laboratories, (Gilroy, Cal.) were used. Homogenates and washed microsomes were obtained as described previously (6, 7) and suspended in 5 mM Tris-HCl, pH 8.0, in 1.15% KCl, before use.

The standard incubation reaction for morphine glucuronide synthesis was composed of various concentrations of morphine sulfate; [*N-methyl*-^{14}C]morphine hydrochloride, 0.228 mCi; various concentrations of UDPGA;[1] $MgCl_2$, 5.0 mM; Tris-HCl, 50 mM, pH 7.6, at 37°; and microsomal protein, 2.0 mg/ml. The total volume was 2.0 ml. Control reactions contained no UDPGA, and zero-time blanks with all reactants present were prepared by adding 1.0 ml of 10% trichloracetic acid immediately after the addition of microsomal protein. Bilirubin solutions were prepared fresh in 0.08 N NaOH. When employed, Triton X-100 was used at 0.05% (v/v), final concentration, and bilirubin was used at 1.0 mM. Reactions were stopped by the addition of 1.0 ml of ice-cold 10% trichloracetic acid and centrifuged. One milliliter of clear supernatant fluid was taken for the analysis of morphine glucuronide formation. Morphine glucuronide was determined as described previously (7).

Microsomal protein was measured using the method of Lowry *et al.* (8).

Michaelis constants were determined using an IBM 360 computer with either the HYPER or SEQUEN program written by Cleland (9, 10, 12).

RESULTS

Bisubstrate kinetic analysis of morphine glucuronidation in native, bilirubin-treated, and Triton X-100-treated liver microsomes. The enzymatic mechanism of microsomal morphine glucuronidation has not previously been studied. Therefore, in order to understand the reaction mechanism of morphine glucuronidation and to elucidate the possible effects of activators, such as bilirubin and Triton X-100, on the mechanism of morphine glucuronidation, bisubstrate kinetic studies were performed.

Results from initial velocity studies of morphine glucuronidation by untreated hepatic microsomes are shown in Fig. 1. Data are graphed as double-reciprocal plots from information obtained from the HYPER computer program of Cleland (9, 10), described by the equation

$$v = VA/K_a + A$$

The intercept on the I/*S* axis and the K_a value depend upon the concentration of the other substrate. These results indicate that studies of enzymatic morphine glucuronidation at a single concentration of either substrate may not provide the best K_a and V_{max} values. Similar relationships have been reported for the glucuronidation of *p*-nitrophenol by hepatic microsomes treated with phospholipase A (11).

Data from Fig. 1 were analyzed by the Cleland computer program designed for sequential reactions (10, 12) according to the equation

$$v = VAB/K_{ia}K_b + K_{ab} + K_bA + AB$$

and plotted in Fig. 2. All lines intersect at a common point at the left of the vertical axis, characteristic of a sequential reaction mechanism. Figure 3 shows results obtained for morphine glucuronidation in the presence of bilirubin at a concentration which activates maximally (0.5 μmol/mg of protein). The characteristic pattern of a sequential reaction mechanism was not changed by bilirubin, but values for K_a and V_{max} were. V_{max} was markedly increased in the presence of bilirubin, and $K_{morphine}$ was increased significantly. However, K_{UDPGA} was not significantly affected.

A summary of these results and those obtained with Triton X-100-treated microsomes are shown and compared with untreated microsomes in Table 1. Triton X-100 treatment was used because it has often been employed as an activator of glucuronyltransferase activity (2, 3). The V_{max} in Triton X-100-treated microsomes was markedly higher than in untreated microsomes. $K_{morphine}$ was about 10 times higher in Triton X-100-treated preparations than in native microsomes. The K_{UDPGA} was not affected significantly in Triton-activated preparations, as seen likewise with bilirubin activation.

Product inhibition studies. In an attempt to gain further information about the reaction mechanism for morphine glucuronide formation, product inhibition

[1] The abbreviation used is: UDPGA, uridine diphosphate glucuronic acid.

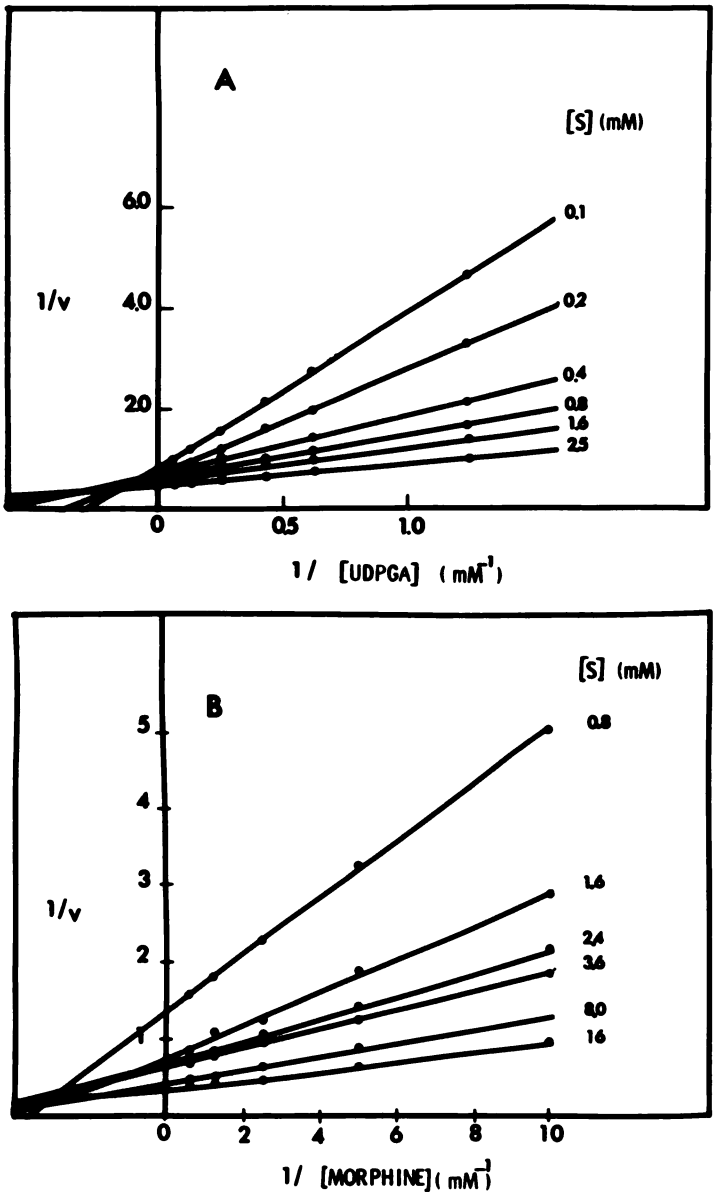


FIG. 1. *Double-reciprocal plots of initial velocities against UDPGA (A) or morphine (B) concentration in the presence of various concentrations of cosubstrate*

Data were obtained from a HYPER program written by Cleland (10).

studies were performed using UDP as the product inhibitor. In experiments with native microsomes, when UDPGA was the variable substrate and the morphine concentration was fixed at 0.8 mM, UDP at 1.0 and 2.0 mM produced inhibition competitive with UDPGA (Fig. 4A). When morphine was the variable substrate and UDPGA was fixed at 6.0 mM, UDP at 1.0 and 2.0 mM produced noncompetitive inhibition (Fig. 4B). Inhibition by UDP competitive with UDPGA in bilirubin-treated microsomes is illustrated in Fig. 5A. A noncompetitive inhibition pattern was observed when morphine was employed as the variable substrate (Fig. 5B).

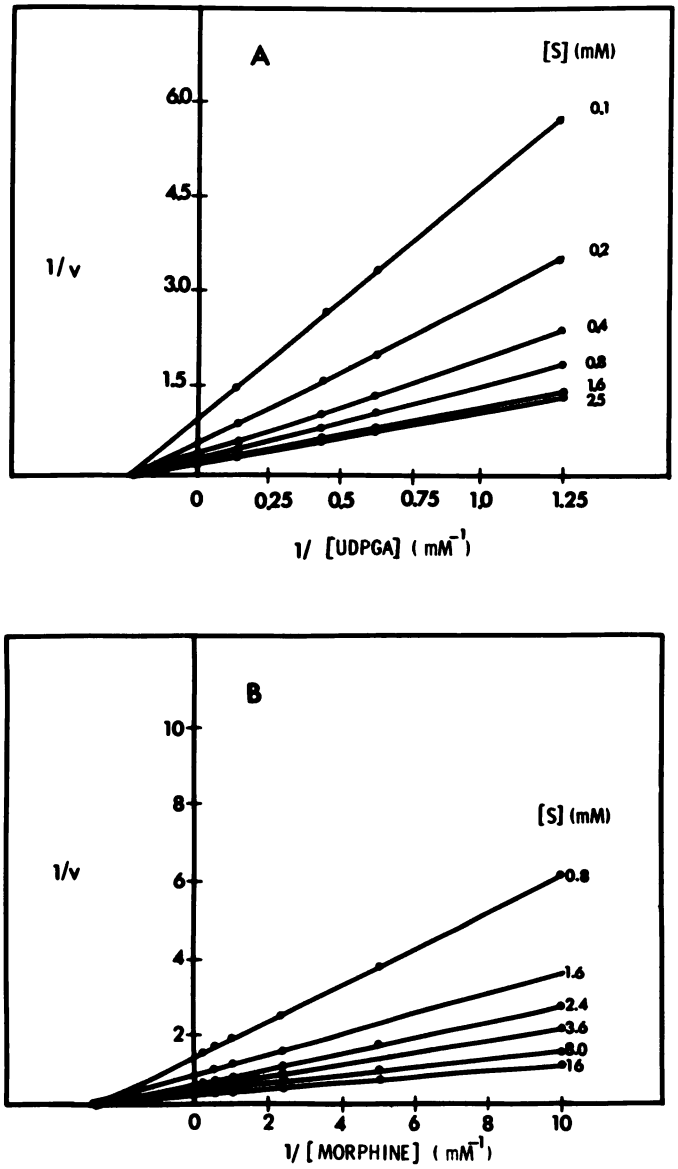


FIG. 2. *Double-reciprocal plots of initial velocities against UDPGA (A) or morphine (B) concentration in the presence of various concentrations of cosubstrate*

Data were obtained using the SEQUEN program written by Cleland (10, 12).

DISCUSSION

Results from kinetic studies show that a plausible mechanism for morphine glucuronidation in hepatic microsomes is a sequential, ordered reaction catalyzed by UDP-glucuronyltransferase. This conclusion is based on the bisubstrate kinetic intersecting pattern for UPGA and morphine and the product inhibition pattern found with UDP. UDP produced inhibition competitive with UDPGA and noncompetitive with morphine. According to these results, we propose the mechanism for the morphine glucuronide reaction with UDP-glucuronyltransferase shown in Scheme 1. This mechanism describes an ordered sequential system. Here UDPGA is the first substrate (*A*) to react with the enzyme,

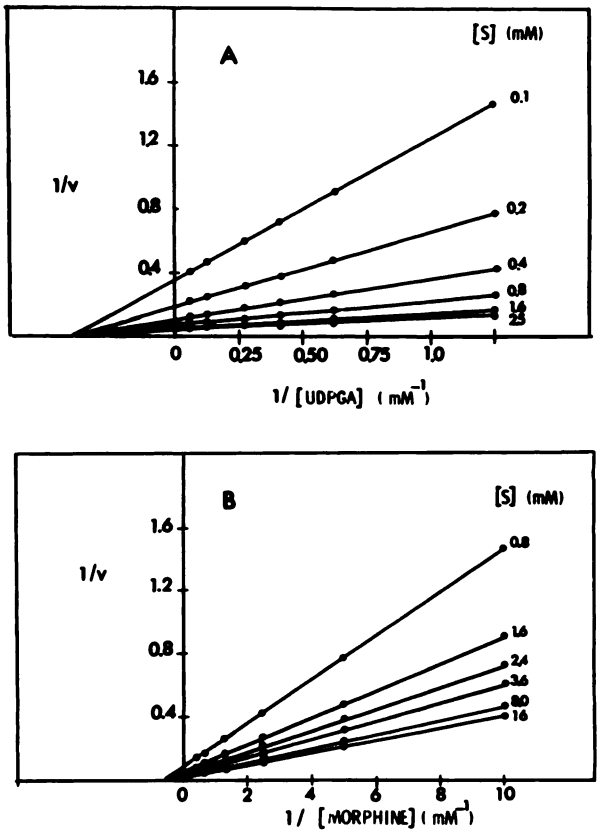


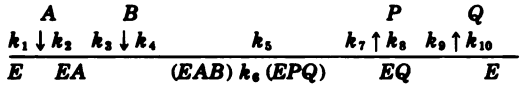
FIG. 3. *Double-reciprocal plots of initial velocities against UDPGA (A) or morphine (B) concentration in the presence of various concentrations of cosubstrate and bilirubin (0.5 μmole/mg of protein)*

TABLE 1

Bisubstrate kinetic constants for morphine glucuronidation in untreated and bilirubin- and Triton X-100-treated liver microsomes

Microsomes	V	K_{UDPGA}	$K_{morphine}$	$K_{i\ UDPGA}$	$K_{i\ morphine}$
Untreated	4.0 ± 0.4	3.50 ± 0.70	0.30 ± 0.1	5.5 ± 2.6	0.4 ± 0.2
Bilirubin	34.0 ± 1.70	1.50 ± 0.30	1.10 ± 0.1	2.60 ± 0.50	1.90 ± 0.50
Triton X-100	20.3 ± 5.40	1.8 ± 0.70	3.0 ± 1.2	0.40 ± 0.30	1.30 ± 0.50

V represents nmoles morphine glucuronide/mg protein/min.
K values are expressed in mM.



SCHEME 1

and morphine is the second substrate (B); morphine glucuronide is the first product (P), and UDP is the second product (Q). A similar reaction mechanism has been described for bilirubin (13). A sequential, random-order mechanism has been described for *p*-nitrophenol glucuronidation (11).

From values for V, K_a, and K_i (Table 1) and from assumptions that

$$k_1 = V/E_t \times \frac{1}{K_{a\ UDPGA}}$$

$$k_2 = V/E_t \times \frac{1}{K_{i\ UDPGA}} K_{a\ UDPGA}$$

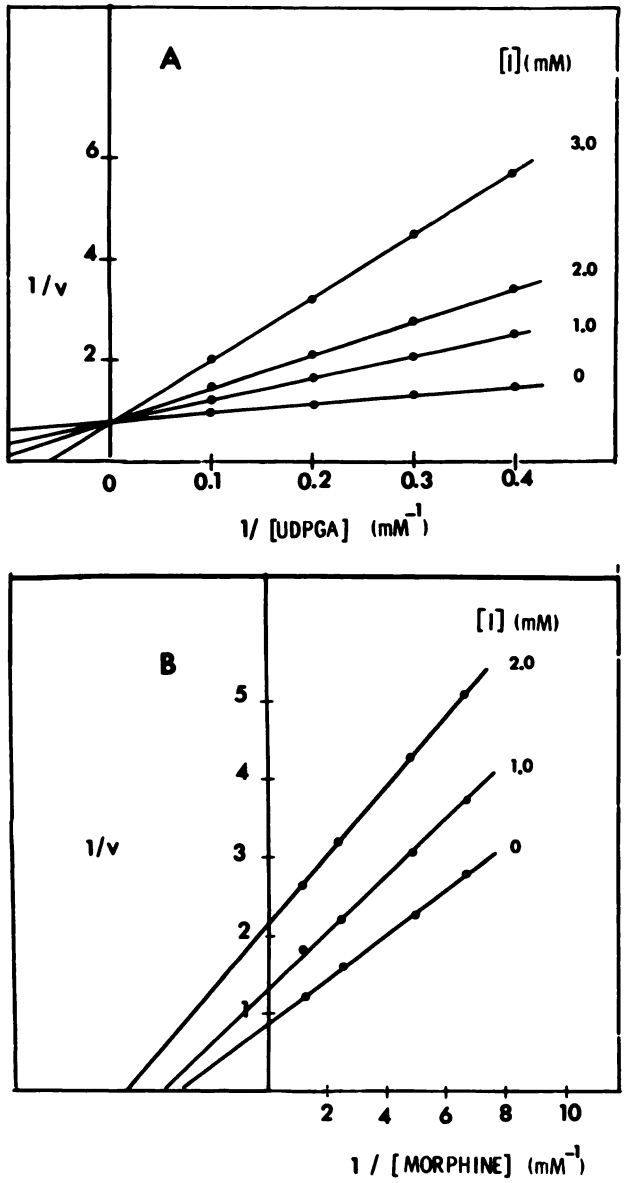


FIG. 4. *Product inhibition by UDP in untreated microsomes*

Double-reciprocal plots of initial velocities against UDPGA (A) or morphine concentration (B), in the presence of various concentrations of UDP and 0.8 mM morphine sulfate (A) or 6.0 mM UDPGA (B), are shown.

it is possible to approximate values for k_1 and k_2. Although the turnover number (V/E_t) cannot be given at this time, the equations can be rewritten as

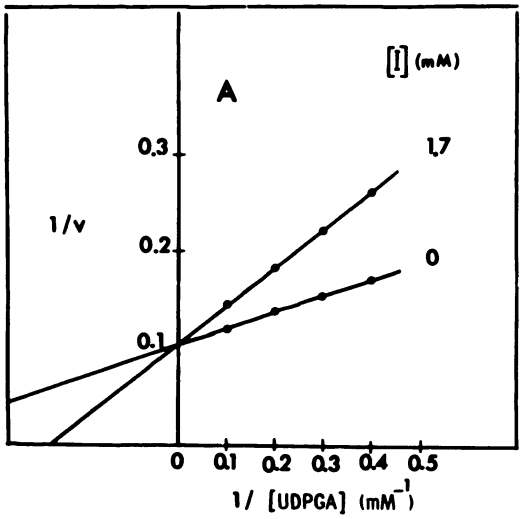
$$k_1E_t = V \times 1/K_{UDPGA}$$

and

$$k_2E_t = V \times K_{i\ UDPGA}/K_{UDPGA}$$

The values for k_1 and k_2 are estimated in Table 2 and represent a reasonable comparison of the effects of bilirubin and Triton X-100 on the mechanism of activation. More work will be needed to verify these observations.

Recently we have solubilized and resolved morphine:UDP-glucuronyltransfer-



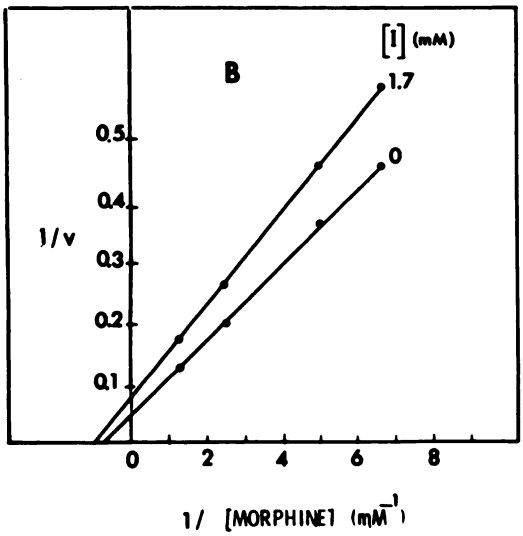


FIG. 5. *Product inhibition by UDP in bilirubin-treated microsomes*

Double-reciprocal plots of initial velocities against UDPGA (A) or morphine concentration (B), in the presence of 1.7 mM UDP and 0.8 mM morphine sulfate (A) or 6.0 mM UDPGA (B), are shown.

ase from hepatic microsomes using Emulgen 911, a nonionic detergent (14). This enzyme preparation had no activity with *p*-nitrophenol, in substantiation of previous studies from this laboratory in which microsomes were used (6). In the solubilized preparation bilirubin had no activating effect (14). This would indicate that bilirubin exerts its activating effect by altering somehow the enzyme in the phospholipid environment in the microsome. Bilirubin differs from the detergent, however, because it has no capacity for solubilizing the enzyme. Therefore bilirubin has certain characteristics which may be useful in situations when activation is desired without release of the enzyme from the membrane. This might be the case *in vivo*, when bilirubin has been found to promote the over-all rate of glucuronide excretion in rats treated with morphine and *p*-nitrophenol (2).

TABLE 2

Effect of bilirubin and Triton X-100 on velocity constants

Microsomes	k_1	k_2
Untreated	1.1	6.3
Bilirubin	23	59
Triton X-100	11.3	4.5

k_1 is expressed in micromoles/min/l.
k_2 is expressed as nanomoles/min.

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