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SHORT COMMUNICATION

Morphine Metabolism

III. Solubilization and Separation of Morphine and *p*-Nitrophenol Uridine Diphosphoglucuronyltransferases

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SUMMARY

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The separation of morphine and *p*-nitrophenol UDP-glucuronyltransferases from rat liver microsomes is described. Hepatic microsomes were solubilized using Emalgen 911, a non-ionic polyoxyethylene nonylphenyl ether, in the presence of glycerol and dithiothreitol, and chromatographed on DEAE-cellulose. The elution of three peaks of transferase activity was observed. One peak displayed transferase activity with *p*-nitrophenol as substrate, one exhibited activity with morphine as substrate, and one displayed activity with either substrate. Neither substrate inhibited the metabolism of the other. Michaelis constants determined for the partially purified preparations were similar to those obtained for native microsomes. These results demonstrate distinct and separate activities for the glucuronidation of *p*-nitrophenol and morphine in rat hepatic microsomes.

This laboratory has been concerned with the mechanisms involved in the formation of morphine glucuronide in the animal organism and has recently reported on the characteristics of certain UDP-glucuronyltransferases in rat hepatic and intestinal microsomes (1, 2). Although indirect evidence indicates that different UDP-glucuronyltransferases catalyze the glucuronidation of morphine and *p*-nitrophenol (1) as well as of several other substrates (3-6), it has not been possible to proceed further in establishing that there are separate UDP-glucuronyltransferases without a method which could provide for the isolation of these enzymes for animal tissues. Others have been able to separate *N*- and *O*-glucuronyltransferases (7, 8) but no effective method has been reported for the isolation of *O*-glucuronyltransferases.

Recently several procedures have been shown to be successful in resolving microsomal cytochrome P-450 and other components of the hepatic mixed-function oxidase system (9-12). In studies designed to develop a method to separate morphine and *p*-nitrophenol glucuronyltransferase activities, we have modified a technique described by Sato *et al.* (13) which was developed for the isolation and purification

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of cytochrome P-450 from other components of the hepatic mixed-function oxidase system. This report describes a procedure useful for the solubilization and isolation of distinct morphine and *p*-nitrophenol glucuronyltransferases and presents certain characteristics for the enzymes resolved from rat hepatic microsomes.

The rate of morphine glucuronidation was determined as described by Del Villar *et al.* (2), and the rate of *p*-nitrophenol glucuronidation was estimated by the method of Isselbacher *et al.* (7). Standard incubation mixtures (final volume, 2 ml) contained enzyme, 50 mM Tris-HCl (pH 8.0), 5.0 mM $MgCl_2$, 5.0 mM UDP-glucuronic acid and either 1.5 mM morphine sulfate (calculated as the free base), with 0.212 μCi of *N*-methyl[^{14}C]morphine hydrochloride, or 1.5 mM *p*-nitrophenol. When alternate substrate inhibition was studied, 10 mM UDP-glucuronic acid was included in reaction mixtures. Reactions were carried out under conditions that gave a rate of product formation linear with time and protein concentration. Protein was measured by the method of Lowry *et al.* (14).

Glucuronyltransferases were isolated from livers of male Sprague-Dawley rats (200–250 g). After 16 hr of fasting, the animals were decapitated and their livers were immediately perfused with ice-cold 0.9% NaCl. Hepatic microsomes were prepared (15), washed with 1.15% KCl, and stored at −80° until used. The microsomal preparation, at a final protein concentration of 4.0 mg/ml, was suspended and solubilized in 0.1 M Tris-acetate buffer, pH 7.5, containing 0.5% (v/v) of the non-ionic detergent Emalgen 911 (Kao-Atlas, Ltd. Tokyo), 25% (v/v) glycerol, and 0.1 mM dithiothreitol. The detergent is a polyoxyethylene nonylphenyl ether. The preparation was held for 30 min at 0° under N_2 and then centrifuged at 105,000 × *g* for 4 hr, resulting in the formation of a small pellet. Glucuronyltransferase activity in the supernatant fraction was unchanged when compared with that of the original microsomal suspension. Because Emalgen 911 interfered with protein determinations, the supernatant fraction was chromatographed twice on a Sephadex LH-20 column (25 × 2.5 cm) in order to remove the detergent. A buffer solution of similar composition but without Emalgen was used to elute the protein from the column. The enzymatic activity was found only in the large protein peak eluted with the void volume. This procedure resulted in the recovery of all the morphine and *p*-nitrophenol glucuronyltransferase activities and in the removal of Emalgen 911 as judged by the disappearance of interfering material in the measurement of protein. The exact amount of Emalgen 911 remaining cannot be determined at this time. The eluted protein was chromatographed on a DEAE-cellulose column that had been equilibrated with 0.1 M Tris-acetate buffer, pH 7.5, containing 25% glycerol and 0.1 mM dithiothreitol. The elution was conducted using a linear KCl concentration gradient (0–0.5 M) in the same buffer.

A typical protein elution pattern and profile of localization of enzymatic activities are illustrated in Fig. 1. Under these experimental conditions three peaks of UDP-glucuronyltransferase activity were eluted at KCl concentration of about 0.1, 0.2, and 0.3 M. Peak I displayed enzymatic activity only in the presence of *p*-nitrophenol as a substrate. Peak III showed glucuronyltransferase activity in the presence of morphine, and peak II was active with either substrate. The most active fraction in peak I had a specific activity of 24 nmoles of *p*-nitrophenol conjugated per milligram of protein per minute, a value 8 times that of the starting material. The most active fraction in peak II had a specific activity of 2.5 nmoles of morphine conjugated per milligram of protein per minute, a value 2.2 times that of the starting material. Maximal values of 20 nmoles of *p*-nitrophenol conjugated per milligram of protein per minute and 2.0 nmoles of morphine conjugated per milligram of protein per minute were found in peak II. Activities of these peaks were stable after storage at 4° for 6 weeks.

Experiments were conducted to determine certain properties of the enzymatic activity eluted from the DEAE-cellulose column. Pooled fractions corresponding to

the activities for morphine (peak III) and *p*-nitrophenol (peak I) glucuronyltransferases were concentrated by ultrafiltration, using a Diaflo PM 30 membrane filter (Amicon). The pooled fractions from peak I displayed no activity toward morphine, and the pool from peak III showed no transferase activity in the presence of *p*-nitrophenol. Furthermore, *p*-nitrophenol (1.5 mM) did not inhibit morphine glucuronidation obtained with the pooled fractions from peak III. These results are consistent with observations made previously when studies were conducted using microsomal preparations (1), and show the high degree of specificity of enzymatic activities separated under this procedure. Table 1 shows a summary of the degree of purification and recovery achieved thus far.

Previous studies from this laboratory showed that bilirubin activates microsomal morphine and *p*-nitrophenol glucuronidation (16). However, bilirubin (1.0 mM) neither activated nor inhibited mor-

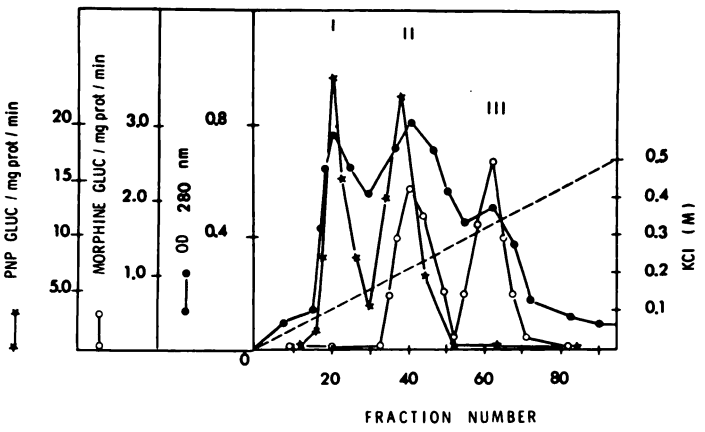

FIG. 1. *Separation of morphine and p-nitrophenol UDP-glucuronyltransferase activities by DEAE-cellulose chromatography*

Solubilized microsomal protein (360 mg) was applied to a column (2.5 × 55 cm) of DEAE-cellulose that had been equilibrated with 0.1 M Tris-acetate buffer, pH 7.5, containing 25% glycerol and 0.1 mM dithiothreitol. The proteins were eluted with the equilibration buffer, which contained KCl in a gradient concentration from 0 to 0.5 M. Each fraction contained 8.0 ml. All procedures were carried out at 0-4°. Morphine and *p*-nitrophenol (PNP) glucuronide (GLUC) formation was measured as described in the text.

TABLE 1

Partial purification of UDP-glucuronyltransferases

Microsomes were solubilized and DEAE-cellulose fractions were obtained as described in the text. Peak I corresponds to pooled samples from tubes 18-21 (Fig. 1), which exhibited glucuronyltransferase activity only in the presence of *p*-nitrophenol. Peak II corresponds to pooled samples from tubes 35-45 (Fig. 1), which displayed enzymatic activity in the presence of morphine and *p*-nitrophenol. Peak III corresponds to the eluates of tubes 58-63 (Fig. 1), which displayed activity only in the presence of morphine.

Enzyme preparation	Total protein	Total enzymatic activity		Specific activity	
		Morphine	*p*-Nitrophenol	Morphine	*p*-Nitrophenol
	mg	*nmoles glucuronide formed/ min*		*nmoles glucuronide formed/ min/mg protein*	
Solubilized microsomes	360	410	1080	1.14	3.0
DEAE-cellulose					
Peak I	25.6	0	460	0	18
Peak II	61.6	105	924	1.7	15
Peak III	28.8	71	0	2.5	0

phine glucuronyltransferase activity from peak III. Therefore, once the microsomal preparation is solubilized and partially purified, bilirubin is no longer capable of activating morphine glucuronidation. This result suggests that bilirubin, like detergents, produced activation of UDP-glucuronyltransferase through an action only on the enzyme as it exists in the native microsomes, since the effect was not observed in enzyme preparations once these structures had been altered through solubilization and purification techniques.

Kinetic studies were carried out using enzyme preparations obtained from peaks I and III. Table 2 shows the Michaelis constants for *p*-nitrophenol, morphine, and UDP-glucuronic acid for these semipurified fractions as well as for the original microsomal suspension. The K_m values obtained from peak I and III preparations are in general agreement with values obtained with microsomal preparations.

Previous studies conducted with hepatic microsomes suggested that two different UDP-glucuronyltransferases catalyze the glucuronication of morphine and *p*-nitrophenol (1). These conclusions were drawn from experiments which showed differential inhibition, lack of alternate substrate inhibition, and differential enhancement of morphine and *p*-nitrophenol glucuronidation by chronic phenobarbital and 3-methylcholanthrene treatment of rats. The current report shows that morphine and *p*-nitrophenol glycuronyltransferase activities can be separated upon solubilization with Emalgen 911 and chromatography on DEAE-cellulose and that morphine and *p*-nitrophenol are conjugated by separate enzymes. Each enzymatic fraction retains characteristics similar to those present in the native microsomal preparation. Future experiments utilizing these semipurified enzyme preparations are planned to develop an understanding of the multiplicity and specificity of the family of glucuronyltransferases in hepatic endoplasmic reticulum.

Other methods have been tested in this laboratory to separate morphine and *p*-nitrophenol glucuronyltransferase activities. Although slight purification was obtained using ammoinum sulfate fractionation, Sephadex filtration, and DEAE-Sephadex chromatography of sonicated or deoxycholate-treated microsomal preparations, these procedures have not been useful for the separation of the enzymatic activities.

The coexistence of both morphine and *p*-nitrophenol glucuronyltransferases in peak II cannot be explained at this time, but will be the subject of further study. Several possibilities come to mind which may explain these results. First, there may be several types of glucuronyltransferases, one of which exhibits a broad substrate specificity (peak II), and others which are quite specific for single substrates (peak I and III). Second, complete solubilization may not have been obtained under the reported experimental conditions, thus resulting in an overlapping of enzymatic activity for the two substrates. The second possibility seems more likely, since *p*-nitrophenol (1.5 mM) did not inhibit morphine glucuronidation catalyzed by the activity obtained in peak II and, similarly, morphine did not inhibit the *p*-nitrophenol glucuronication activity of peak II. The

TABLE 2

Michaelis constants of UDP-glucuronyltransferase activities

All incubation media contained 50 mM Tris-HCl (pH 8.0), 5.0 mM $MgCl_2$, and 4.0 mg of microsomal protein or 1.5 mg of protein from concentrated peaks, in a final volume of 2.0 ml. When the K_m for morphine or *p*-nitrophenol was determined, the final concentration of UDP-glucuronic acid was 5.0 mM and the final concentration of morphine ranged between 0.125 and 2.5 mM. When the K_m for UDP-glucuronic acid was determined using morphine as acceptor, the final concentration of morphine was 1.5 mM and the final concentration of UDP-glucuronic acid ranged between 1.0 and 12 mM. Results were analyzed using an IBM 360 computer with the HYPER program written by Cleland (17).

Enzyme preparation	K_m		
	Morphine	*p*-Nitrophenol	UDP-glucuronic acid
	mM	*mM*	*mM*
Microsomes	0.3	1.1	3.5
Peak I or III	0.5 (III)	0.6 (I)	4.0 (III)

most reasonable explanation of the dual substrate activity of peak II may be the incomplete dissociation of both the morphine- and *p*-nitrophenol-specific transferases from other proteins and membrane fragments in the original Emalgen-treated microsomal preparation.

These results clearly indicate that glucuronyltransferase activity is attributable to more than one enzyme entity. Although we have demonstrated the existence of two separate enzymes, it is very likely that there are present in the endoplasmic reticulum additional enzymes with glucuronyltransferase activity specific for other substrates.

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