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(23) If $K_i$ values are determined by suitable replotting of the slopes and intercepts of double-reciprocal plots vs. inhibitor concentration,[24] the inhibition constant for the slopes ($K_{is}$) is not equal to that for the intercepts ($K_{ii}$). However, we have chosen to follow the suggestion of Butterworth[25] and retain the use of Dixon plots for determining $K_i$ values in conjunction with Lineweaver–Burk plots to determine the type of inhibition.
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# Immobilized Glucuronosyltransferase for the Synthesis of Conjugates[1]

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Partially purified rabbit liver UDPglucuronosyltransferase is immobilized on agarose by the cyanogen bromide activation method. Both soluble and matrix-bound enzyme preparations display very similar $K_m$ and pH optimum. The storage stability of the immobilized enzyme at 4° is 5–10 times improved over the soluble preparations. The agarose-bound UDPglucuronosyltransferase is successfully used in the synthesis of *p*-nitrophenyl glucuronide in an overall yield of 50–70%. The matrix-bound enzyme is reusable over an extended period of time and offers an easy and convenient synthetic tool for various drug glucuronides.

The general progress in the understanding of drug metabolism has been hindered to a certain extent by the paucity of pure glucuronides of known structure available for use as standards. Isolation of drug glucuronides from dosed rabbits and from overdosed patients has been in certain cases only moderately successful.[2a] Attempts have also been made to synthesize selected drug glucuronides by in vitro enzymatic synthesis using crude liver homogenate or fresh liver slices.[2b] Direct chemical syntheses of glucuronides have experienced great difficulties especially in cases of polyfunctional drug molecules. The present study indicates that the use of immobilized UDPglucuronosyltransferase (E.C. 2.4.1.17) on an inert matrix offers good potential for the synthesis of glucuronides of various drugs.

The micrososomal enzyme, UDPglucuronosyltransferase, catalyzes the transfer of glucuronic acid from uridinediphosphoglucuronate to a wide variety of drugs and hormones.[3] It is not known whether the glucuronosyltransferase activity resides in one or several enzymes with overlapping specificities.[4] Although it is primarily a hepatic enzyme, it has been found in various other tissues of rat, rabbit, and guinea pig.[5] UDPglucuronosyltransferase is one of the least understood of the mammalian metabolic enzymes. The free enzyme is very sensitive to even moderately high temperature. Because of its limited stability, the earlier isolation methods,[6–8] requiring several days, resulted in great losses in enzymatic activity. A detailed comparative study of the stability and specific activity of the enzyme in different species has not been undertaken. The enzme has been solubilized and partially purified from various mammalian tissues.[6–8] Although the enzyme is not available in pure form, its various properties, including pH optimum, substrate specificities, gel filtration characteristics, and various kinetic parameters (in soluble form), have been extensively studied. The enzyme has never been immobilized on a solid support though a large number of methods are available.[9,10]

During recent years numerous applications of immobilized enzymes have been experimentally realized. It has been shown that immobilized enzyme reactors have various advantages compared to the corresponding enzymatic reactions in solution.[11] Immobilized preparations of glucose oxidase and urate oxidase have been elegantly used in the automated determination of glucose[12] and uric acid.[13] Proteolytic enzymes like trypsin and papain have been immobilized.[14] This paper reports covalent immobilization of UDPglucuronosyltransferase to agarose by the CNBr activation method and its application to the synthesis of model drug glucuronides.

## Experimental Section

**Materials and Methods.** Agarose (Sepharose 4B) was purchased from Pharmacia. Uridinediphosphoglucuronic acid ammonium salt and *p*-nitrophenol glucuronide were obtained from Sigma. The solvents and reagents were analytical grade and were

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used without further purification. The nonionic detergents were purchased from Savco, Ft. Lauderdale, Fla. Silica gel GF thin-layer plates were obtained from Analtech Inc. The thin-layer chromatograms were run with a homogeneous mixture of 1-butanol–acetic acid–$H_2O$ (35:3:10). The spots were made visible with a naphthoresorcinol spray as follows. The spray reagent consisted of a mixture of 5 vol of 0.2% naphthoresorcinol in acetone and 1 vol of 10% (v/v) aqueous phosphoric acid. After spraying the reagent the plate was heated to 80–100° for 5 min. The glucuronide becomes visible as a blue-purple spot with a pink background.

**Enzyme Isolation.** UDPglucuronosyltransferase was isolated from rabbit liver by modification of the described procedure.[8] All steps in the isolation procedures were performed at 4° unless stated otherwise. Fresh rabbit liver was minced and homogenized (Polytron) as 25% w/v in 0.01 *M* borate–phosphate buffer, pH 8.0, containing 0.14 *M* KCl for 2 min at setting 3. The homogenate was centrifuged at 200g (Sorvall Model DC-2) for 20 min. The supernatant was further centrifuged at 100000g (Beckmann ultracentrifuge Model L2-65B) for 1 hr. The pellet was resuspended (with the help of Polytron, if necessary) in the homogenizing buffer containing 0.1% Lubrol-PX[15] in a volume equal to the volume of the initial homogenate. The suspension was sonicated (Insonator), while in an ice bath, for ten bursts of 10 sec each with 50-sec intervals between each burst. The sonicated material was recentrifuged at 100000g for 1 hr. Almost 80% of the enzymatic activity was present in the latter supernatant. The solublized enzyme preparation was used in the work described here without further purification of the enzyme.

**Enzyme Assay.** *p*-Nitrophenol[16] was used to assay the enzymatic activity of the soluble enzyme as well as the immobilized enzyme. A stock solution of *p*-nitrophenol was prepared in ethanol, which was then diluted to 0.15 m*M* with 0.05 *M* Tris buffer, pH 8.0, so that the resulting solution contained 0.5% ethanol. A typical assay incubation consisted of 0.5 ml of a 1:1 suspension (in 0.05 *M* Tris buffer, pH 8.0) of the agarose-bound enzyme, 1.5 ml of 0.05 *M* Tris buffer, pH 8.0, containing 0.15 m*M* *p*-nitrophenol, 0.4 m*M* UDPGA, and 0.5% ethanol. The incubations were generally performed in 12-ml screw-cap polypropylene tubes with mild shaking (wrist action mechanical shaker, approximately 40 oscillations per minute) for 60 min at 25°. The reaction was stopped by addition of 8.0 ml (four times the incubation volume) of 0.05 *M* glycine buffer, pH 10.4. The contents were centrifuged at 2000g for 5 min and the decrease in absorbence of the supernatant at 400 nm was determined with a Zeiss spectrophotometer. The control incubations were performed in the identical manner except that the agarose–enzyme conjugate was replaced with the same volume either of plain unsubstituted agarose or of the buffer.

**Immobilization of UDPglucuronosyltransferase to Agarose.** The dialyzed enzyme was coupled to agarose by the modified cyanogen bromide method.[17] Due to the relative instability of the enzyme, it was coupled to agarose soon after being dialyzed. The dialyzed enzyme preparation (40 ml, 3.7 mg of total protein per milliliter) was coupled to 50 ml of CNBr-activated agarose at 4° for 6 hr. After coupling the agarose was washed with 5 vol of 0.1 *M* $NaHCO_3$, pH 8.0, and incubated at 4° for 4 hr with 1 vol of 0.1 *M* aqueous ethanolamine, pH 8.0. The conjugated agarose was then extensively washed under suction with 0.1 *M* $NaHCO_3$, pH 8.0 (approximately 10 vol over 30 min), until no protein was detected (absorbance at 280 nm) in the washings. Finally the conjugated agarose was washed with 2–3 vol of 0.05 *M* Tris buffer, pH 8.0, and stored at 4° in the latter buffer as a 1:1 suspension. The substitution on agarose was found to be approximately 3.0 mg of proteins per milliliter of packed gel as determined by difference in total protein content by the method of Lowery et al.[18] before and after coupling. The agarose-immobilized enzyme so obtained was used in all the kinetic experiments as well as for the glucuronide synthesis.

**Enzymatic Synthesis of Drug Glucuronides.** Phenolic glucuronide of *p*-nitrophenol as a model drug was prepared using the agarose-bound UDPglucuronosyltransferase. The agarose–enzyme conjugate (80 ml settled gel volume) was treated with 120 ml of 0.8 m*M* drug (containing 1% ethanol) and 120 ml of 2.0 m*M* UDPGA, both in 0.05 *M* Tris buffer, pH 8.0. The suspension was mildly shaken (wrist action shaker, 40–50 oscillations per minute) for 6 hr at room temperature. The gel was filtered and washed under suction with 3 × 80 ml of 0.05 *M* Tris, pH 8.0. The combined filtrates were flash evaporated (30–35°) to about 50 ml and acidified to pH 4.5 with 1 *M* HCl. The aqueous solution was extracted five times with 50 ml of water-saturated 1-butanol. The combined butanol extracts were flash evaporated to dryness. The overall synthetic yields of *p*-nitrophenol glucuronide varied between 50 and 70%, determined spectrophotometrically.

Table I. Effect of Various Treatments on the Solubilization of Membrane-Bound UDPglucuronosyltransferase[a]

| Treatment | Rel act., %, in pellet | Rel act., %, in supernatant |
|---|---|---|
| Untreated | 100 | 0 |
| Lubrol-PX | 43 | 0 |
| Lubrol + sonication | 86 | 100 |
| Lubrol-PEX[b] | 0 | 0 |
| Lubrol-MOA[b] | 0 | 0 |
| Triton-X100[b] | 0 | 0 |
| 2-Chloroethanol[b] | 0 | 0 |
| Glycerol | 0 | 0 |
| Glycerol + sonication | 69 | 41 |
| Sonication alone[c] | | |
| 20 × 10 sec | 41 | 0 |
| 10 × 10 sec | 59 | 48 |
| 5 × 10 sec | 69 | 24 |

[a] The 200g supernatant (5 ml) of rabbit liver homogenate (see text) was treated with various reagents in a final concentration of detergents, 0.1% w/v; glycerol and 2-chloroethanol, 1.0% v/v. The samples were centrifuged (100000g, 1 hr) directly or after sonication at 4° for ten bursts of 10 sec each with 50-sec intervals between each burst. The enzymic activity present in the pellet as well as the supernatant was determined as described in the text. [b] These samples did not show any enzymic activity in the pellet or the supernatant even after sonication. [c] With 50-sec intervals between each burst of sonication.

## Results

**Solubilization of the Enzyme.** As the hepatic UDPglucuronosyltransferase is membrane bound, optimum conditions were sought to solubilize the enzyme with minimum loss in activity. Various nonionic detergents and other conventional solubilizing agents were evaluated for this purpose. The proteins which do not settle down under a centrifugal force of 100000g within 1 hr were considered as "soluble proteins". Table I shows that Lubrol-PX in combination with controlled sonication was the best condition tested to solubilize this enzyme when it was isolated from mammalian liver. Lubrol-PX and other nonionic detergents tested alone at the concentration of 0.1% were not effective to solubilize the enzyme. Sonication alone had also limited success. The use of Lubrol-PX at higher or lower concentrations than 0.1% was not suitable, producing either inactivation or diminished solubilization of the enzyme.

**pH–Activity Profile.** The immobilized UDPglucuronosyltransferase displayed a relatively sharp activity optimum at pH 8.0 when *p*-nitrophenol was used as substrate (Figure 1). The pH optimum was unchanged when 0.5–5% ethanol was present in the incubating medium. The pH optimum of the soluble enzyme using *p*-nitrophenol as the substrate was also found to be 8.0.

**Kinetic Parameters.** The agarose-immobilized enzyme exhibited a linear time course (Figure 2) over 2-hr period when 2 ml of the conjugated enzyme (6 mg of total proteins) was incubated in the presence of 0.15 m*M* *p*-nitrophenol and 1.0 m*M* UDPGA in 0.05 *M* Tris, pH 8.0. Neither substrates had detectable inhibitory effects even at 20-fold higher concentrations. The kinetic measurements were performed at 25° with 45-min incubation time

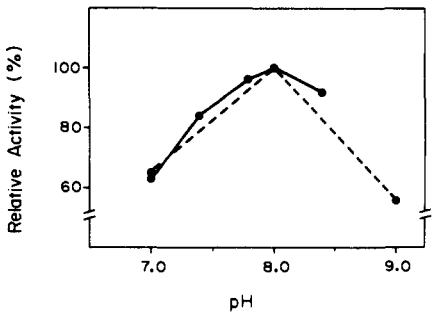


Figure 1. The pH–activity relationship of the solubilized and the agarose-bound UDPglucuronosyltransferase. The enzymic activities were determined in various buffer solutions after incubation at 25° for 60 min as described in the text. Each point on the curve represents the mean value of triplicate incubations.

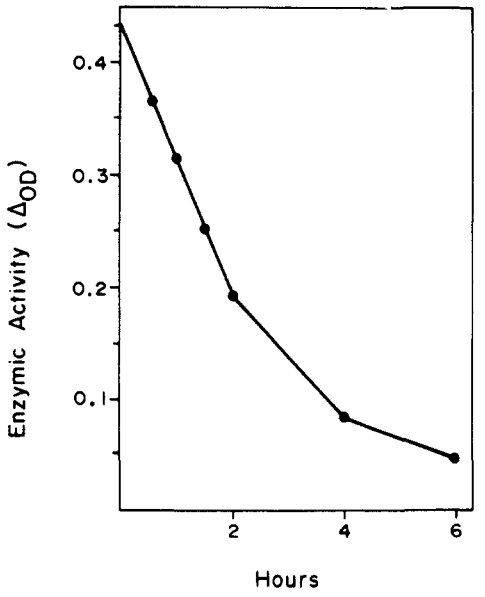


Figure 2. Time course of immobilized UDPglucuronosyltransferase. The incubation mixture consisted of 4 ml of a 1:1 suspension of the agarose-bound enzyme, 12 ml of 0.15 m*M* *p*-nitrophenol in 0.05 *M* Tris, pH 8.0, containing 0.5% ethanol, and 0.4 m*M* UDPGA. The incubations were performed in screw-cap polypropylene vials at 25° with mild shaking. Aliquots of 1.0 ml were taken out at various times and immediately treated with 4.0 ml of 0.05 *M* glycine buffer, pH 10.4. The assays were performed as described in the text. The values on the ordinate represent relative fall in the absorbance at 400 nm.

using 0.15 m*M* *p*-nitrophenol and 1.0 m*M* UDPGA in 0.05 *M* Tris, pH 8.0. Two enzyme concentrations were used to produce the two lines in the double reciprocal plot (Lineweaver–Burke plot, Figure 3). The concentration of the enzyme was varied by diluting the enzyme-bound agarose with an equivalent amount of unsubstituted agarose. In this way, for either enzyme concentration, there would be an equal volume of agarose beads in the incubation mixture. The $K_m$ of *p*-nitrophenol for the agarose-bound enzyme was $6.6 \times 10^{-4}$ as obtained from this double reciprocal plot (Figure 3). The $K_m$ values for the soluble enzyme in the literature vary between $6.2 \times 10^{-4}$ and $2.8 \times 10^{-5}$ *M* for *p*-nitrophenol.[6,19]

**Stability Parameters of the Immobilized Enzyme.** The enzyme displays a pronounced "stabilization" upon covalent immobilization on agarose matrix. The half-life for the free enzyme (in solution) stored at 4°, 0.05 *M* Tris, pH 8.0, in a polyethylene container is less than 10 days, while the agarose-bound enzyme under similar conditions has a half-life of more than 45 days.

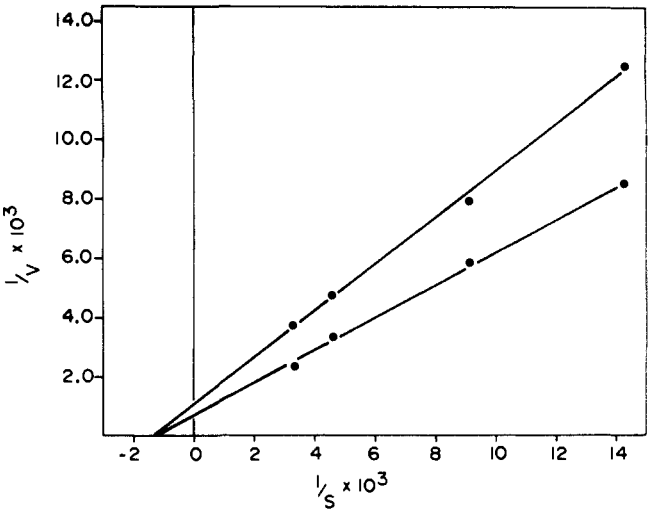


Figure 3. Double reciprocal plot of the enzymic activity of agarose-bound UDPglucuronosyltransferase with *p*-nitrophenol as substrate. The enzymic activity of 0.5 and 1.0 ml of the agarose conjugate (as a 1:1 suspension in 0.05 *M* Tris, pH 8.0) was incubated (in triplicate) with various concentrations of the substrate in 0.05 *M* Tris, pH 8, containing 0.5% ethanol and 0.4 m*M* UDPGA in a total volume of 4 ml. The incubations were performed as described in the text. The upper line represents incubations that contain one-half the enzyme concentration of the incubations plotted on the lower line. *V* and *S* represent the initial velocity of the enzymatic reaction and the substrate concentration, respectively. Control incubations in triplicate were performed under identical conditions for each substrate concentration in the absence of the enzyme.

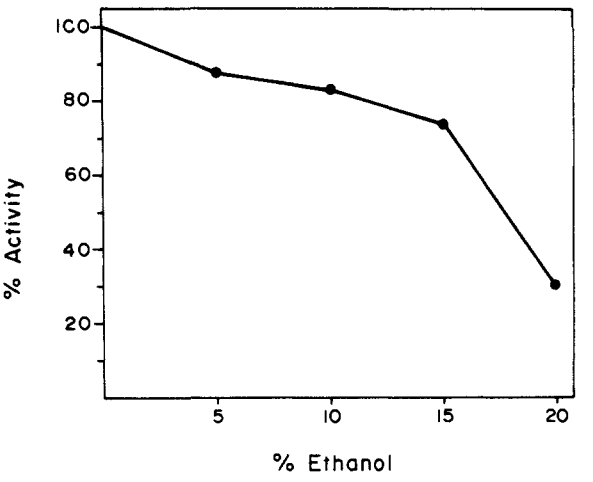


Figure 4. Effect of ethanol on the activity of the agarose-bound enzyme. The enzymic activity was determined at 25° as described in the text except that the buffer contained varying amounts of ethanol. Each incubation was performed in duplicate. Control incubations (also in duplicate) without the enzyme were performed for each ethanol concentration.

Because of the limited solubility of some drugs in aqueous medium, addition of water-soluble organic solvents is necessary for the enzymatic conjugation. The stability of the agarose-bound enzyme was tested with increasing concentrations of four water miscible organic solvents. Figure 4 shows the effect of various concentrations of absolute ethanol on the relative activity of the agarose-bound enzyme. At 20% ethanol concentration more than 50% of the enzymatic activity was lost. The effects of dioxane, dimethyl sulfoxide, or dimethylform-

amide on the enzyme activity were adverse. As little as 2% of dioxane resulted in almost complete loss of enzymatic activity. Albumin and propylene glycol were used with greater success to solubilize substrates for some syntheses (unpublished results).

**Characterization of the Glucuronide.** The synthetic *p*-nitrophenyl glucuronide extracted in butanol was almost homogeneous when tested on silica gel thin-layer chromatography using the solvent system composed of 1-butanol–acetic acid–$H_2O$ (35:3:10). The minor impurity observed was that of unconjugated UDPGA. The TLC spots were visualized with naphthoresorcinol spray. The stereochemistry of the synthetic product was confirmed as a $\beta$-linkage by cleavage of the synthesized *p*-nitrophenyl glucuronide with $\beta$-glucuronidase (Sigma). The enzymatic hydrolysis of the glucuronide was followed spectrophotometrically at 400 nm. The synthetic glucuronide was further characterized by gas chromatographic–mass spectral analysis. An authentic sample of *p*-nitrophenyl glucuronide was used for comparison by TLC and mass spectral analysis with the synthetic conjugate. The glucuronide was converted to a volatile derivative for gas chromatography–mass spectrometric analysis by treatment with bis(trimethylsilyl)trifluoroacetamide as has been described.[2a]

The high mass range of the mass spectrum of tetrakis(trimethylsilyl)-*p*-nitrophenyl glucuronide shows a molecular ion observed at $m/e$ 603, accompanied by M – 15 and M – 105 ions, characteristic of trimethylsilylated glycosides. The base peak at $m/e$ 375 and the peak at $m/e$ 465 correspond to ions characteristic of persilylated glucuronides.[2a] The M – 392 peak characteristic of phenol-linked glucuronides is observed at $m/e$ 211.

## Discussion

In the present study it was necessary to minimize the duration of the isolation process in order to increase the activity of the immobilized enzyme for synthetic applications. The average time elapsed (at 4°) between mincing of the freshly obtained rabbit liver and the immobilization step was reduced to 8 hr.

The solubilized UDPglucuronosyltransferase obtained from rabbit liver was found in our hands to be of far greater stability than that obtained from rat or calf liver.

As is found in other immobilized enzyme preparations, agarose-immobilized UDPglucuronosyltransferase exhibits enhanced stability. Whereas the soluble nonbound enzyme exhibits a half-life at 4°C of only a few days, the immobilized enzyme has a half-life of more than 45 days at 4° and about 1–2 days at 25°. The immobilized enzyme may be used three or four times for syntheses carried out at 25°. At 37° the immobilized as well as the soluble enzyme displays 2–5 times faster rates than at 25°, although the half-life of even the agarose-bound enzyme at 37° is only about 25% of that at 25°.

The CNBr activation method used to couple the enzyme to agarose immobilizes about 90% of the total proteins and virtually 100% of the enzymatic activity present in the coupling medium. The amount of CNBr used per each milliliter of agarose for the activation purpose can be varied between 50 and 150 mg without appreciable affect on total protein coupled or to the activity of the coupled enzyme. The insolubilized enzyme is fairly resistant to moderate concentrations of ethanol, with a 50% reduction in activity occurring when the ethanol concentration reaches 17% (v/v). The agarose-bound enzyme treated with buffers containing 2% dioxane loses all activity and activity cannot be recovered even by extensive washing of the gel with 0.05 *M* Tris, pH 8.0.

The $K_m$ value for *p*-nitrophenol of the agarose-bound enzyme is about the same as that of the soluble enzyme. An inert matrix like that of agarose seems to provide negligible perturbation of the tertiary or quaternary structure of matrix-bound enzyme. Furthermore, the $K_m$ value for *p*-nitrophenol is not affected by the presence of 0.5–5% ethanol.

The general method described here for the synthesis of a model phenolic glucuronide seems to have great potential for the synthesis of various glucuronides otherwise not easily realized through classical methods of in vitro synthesis.

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