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IMMOBILIZED HEPATIC MICROSOMES FOR THE SYNTHESIS OF GLUCURONIDES

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A method has been developed for the direct immobilization of hepatic microsomes. The technique is simple and efficient. The immobilized microsomes retain biological activity and can be used to perform enzymatic reactions.

The immobilization of relatively pure hepatic microsomal enzymes for glucuronidation (1,2) and *N*-oxidation (3) has been reported for the synthesis of metabolites as well as for possible substitution for certain hepatic functions in man. These membrane-bound enzymes must first be solubilized by various techniques using detergents and subsequently be immobilized on inert matrices. The difficulty of solubilizing and purifying these enzymes, however, has restricted their use on a solid phase to only a few groups.

A method for the direct immobilization of hepatic microsomes has been developed in this laboratory. This technique has been highly effective for the synthesis of various drug metabolites.

We are presently concentrating on the synthesis of glucuronides. The glucuronides of 7-*OH*-chlorpromazine, 7-*OH*-prochlorperazine, and 7-*OH*-perphenazine have been isolated in pure form and converted to their respective parent compounds upon treatment with β-glucuronidase. A thin-layer chromatography (TLC) system for these compounds is described in Table 1. Further analysis by NMR and gas chromatography/mass spectrometry is continuing in conjunction with J. C. Craig of the University of California at San Francisco.

While we are still in the process of determining the most efficient method of microsomal fragmentation and isolation, the following method has been proven satisfactory. Rat and hog liver microsomes are prepared and sonicated by a modification of the method described by Mowat and Arias (4). Microsomes are initially isolated by centrifugation at 78,000*g* for

TABLE 1. **Thin-Layer Chromatography of 7-Hydroxychlorpromazine, 7-Hydroxyprochlorperazine, 7-Hydroxyperphenazine, and Their Respective Glucuronides on Silica Gel Plates**[a]

Compound	R_f	Color
7-Hydroxychlorpromazine	0.87	Purple
7-Hydroxychlorpromazine glucuronide	0.16	Pink
7-Hydroxyprochlorperazine	0.70	Purple
7-Hydroxyprochlorperazine glucuronide	0.12	Pink
7-Hydroxyperphenazine	0.86	Purple
7-Hydroxyperphenazine glucuronide	0.20	Pink

[a] Solvent is 9:1:acetone:0.05% ammonia water (*v/v*). A sulfuric acid/ferric chloride spray was used as a developing agent (7).

35 min. The pellet is resuspended and sonicated as described (4). This mixture is then centrifuged at 100,000*g* for 1 h.

The supernatant fraction is mounted on zirconia-clad, 1,350 Å pore diameter glass beads (Pierce Chemical Co.). Glutaraldehyde is used as a carbonyl intermediate and the protein attached by Schiff base coupling. Forty milliliters of 2.5 wt% glutaraldehyde is added to 4 g of glass beads. The mixture is evacuated in a vacuum aspirator for 30 min to remove air bubbles. The vacuum is released and the reaction continued at room temperature for another 40 min. The glutaraldehyde is then decanted and the beads are washed at least ten times with 30-ml water. Microsomes ranging in concentrations of 50 to 25 mg/ml are then added for a total of 25 to 50 mg/g glass beads. Immobilization proceeds at 8°C for 2–12 h.

Typical microsomal glucuronidation activities range from 3 to 5 nmol/min-mg at 37°C and compare well with other data (5) for untreated guinea pig liver microsomes. Typical yields are 15–20 mg microsomes per gram dry beads with activities of 3–5 nmol/min-mg immobilized microsomes at 37°C. The immobilized microsomes are then stored at 4°C and rinsed three or four times a day for a few days in 0.05 M phosphate buffer, pH 7.6. Activity is monitored by assays using *p*-nitrophenol as a glucuronide acceptor (6).

Small amounts (3 to 5 mg) of glucuronides were prepared in batch reactors with continuous shaking in a constant temperature bath. Large amounts (>50 mg) were prepared in a fluidized-bed reactor (3). A typical reaction medium contains 10^{-3} M aglycone, 1.5×10^{-3} M UDPGA (Sigma), 0.4×10^{-4} M phosphate buffer, pH 7.4, and 0.5–2 g active glass beads per 10 ml final solution.

Three to five milligrams of each of the hydroxyphenothiazines listed in Table 1 were incubated and the reactions monitored by TLC. The spots

representing the parent compounds gradually disappeared while their respective glucuronide spots increased in intensity. At 100% conversion, there were no detectable traces of parent compound. Only a single glucuronide spot remained on each chromatogram. The glucuronides were then extracted with *n*-BuOH, brought down to dryness with nitrogen, and reincubated in the presence of bovine liver β-glucuronidase. These incubations were also monitored by TLC. The glucuronide spots gradually disappeared while their parent compounds increased in intensity. At 100% conversion, there were no detectable traces of glucuronide.

An extraction technique has been developed to separate 7-*OH*-CPZ from its glucuronide. The parent compound is extracted twice from an aqueous ammoniacal solution of glucuronide (pH 11–13) with $CHCl_3$. After a water backwash, both the 7-*OH*-CPZ and its glucuronide are pure chromatographically.

Mowat and Arias (4) demonstrate by electron microscopy that their "soluble" protein fraction, even after column chromatography, actually consists of membranous structures 800 to 2000 Å in diameter. They note further the presence of nucleoside phosphatase activity and a similarity of phospholipid content with microsomes, concluding that electron microscopy is advisable before assuming that a membrane-bound enzyme has been made soluble. While we have noted the presence of a microsomal oxidase (3) activity and have been able to use the microsomal pellet fraction directly for mounting, the possibility exists that the activity found on glass may in reality be due to enzymes temporarily solubilized and subsequently immobilized. This question is to be further resolved by electron microscopy.

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