An Improved Assay Technique for Uridine Diphosphate Glucuronosyltransferase Activity towards 5-Hydroxytryptamine and some Properties of the Enzyme

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A simplified and sensitive procedure for the routine assay of UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine (serotonin) was developed and the reaction product confirmed as the *O*-glucuronide of this substrate. The assay was used to study some properties of this UDP-glucuronosyltransferase activity. In mouse liver activity was stimulated by membrane-perturbation procedures and by UDP-*N*-acetylglucosamine. In rat liver it was stimulated by digitonin, but not by diethylnitrosamine. Mouse duodenum, kidney, and lung possessed activity that was less latent than in liver. No activity was found in homogenates of brain. The activity was present in Gunn rat liver, though only one-third of that in Wistar rat liver. Cat liver contained no UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine.

Glucuronidation is a major route of conjugation of toxic endogenous and xenobiotic compounds. The membrane-bound enzyme(s) responsible, UDP-glucuronosyltransferase(s) (EC 2.4.1.17), shows activity towards many substrates, but the properties of these various activities can differ. Although much has been published on the properties of UDP-glucuronosyltransferase and its regulation (see Zakim & Vessey, 1976; Dutton & Burchell, 1977), xenobiotic substrates, such as *p*-nitrophenol, have been used predominantly. Such 'unnatural' substrates are likely to be glucuronidated by one or more of the existing forms of the enzyme, evolved to glucuronidate either endogenous compounds or xenobiotics present in the organism's natural diet, and thus overlap of activities might occur (see Dutton *et al.*, 1976). 'Natural' substrates are therefore more appropriate for studies on the heterogeneity, differential development and purification of the different UDP-glucuronosyltransferase forms.

Commonly used 'natural' substrates include the steroids and bilirubin. This paper describes an improved UDP-glucuronosyltransferase assay with the substrate 5-hydroxytryptamine (serotonin) and reports some properties of this activity of the enzyme.

5-Hydroxytryptamine glucuronide has been isolated from mouse and rat urine (Weissbach *et al.*, 1961) and its formation from labelled aglycone in perfused rat liver has been reported (Tyce *et al.*, 1968a). Thus glucuronidation of 5-hydroxytryptamine in these species is of physiological importance.

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Airaksinen *et al.* (1965) assayed the formation of radioactively labelled 5-hydroxytryptamine glucuronide in UDP-glucuronic acid-supplemented rat liver homogenates by the subsequent chromatography and radioactive scanning of the acetone-insoluble reaction products. In the rapid and simple method described below, the 5-hydroxytryptamine glucuronide formed is isolated by double acetone precipitation and spectrophotometrically measured as a coloured complex with *p*-dimethylaminobenzaldehyde, thus eliminating the chromatography stage and allowing quantification without the use of labelled substrate.

Materials and Methods

Digitonin, *p*-dimethylaminobenzaldehyde, 5-hydroxytryptamine (creatinine sulphate complex and oxalate salts), D-glucaric acid 1,4-lactone, Triton X-100, UDP-glucuronic acid and UDP-*N*-acetylglucosamine were obtained from Sigma Chemical Co., Kingston on Thames, Surrey, U.K., and diethylnitrosamine was from Eastman Kodak Co., Rochester, NY, U.S.A. All other reagents were AnalaR grade from British Drug Houses, Poole, Dorset, U.K.

Animals and tissue preparation

Homogenates of liver, lung, brain, kidney, and duodenum from freshly killed DBA/2 mice were prepared in ice-cold water with a Teflon/glass homogenizer. Homogenate concentrations were 2.5–10% (w/v). Liver homogenates from Wistar and

homozygous Gunn rats and young (10–30 weeks) male cats were prepared similarly. In certain experiments homogenates were progressively sonicated, or preincubated at 37°C, by the methods of Leakey *et al.* (1976) and Burchell *et al.* (1975) respectively.

Chromatography

5-Hydroxytryptamine and its metabolites were chromatographed by the method of Airaksinen *et al.* (1965) on Whatman no. 1 paper and developed with butan-1-ol/acetic acid/water (4:1:5, by vol., organic phase only). Indoles were detected by spraying with a 20% (v/v) solution of Ehrlich's reagent [10% (w/v) *p*-dimethylaminobenzaldehyde in conc. HCl] in acetone.

Enzyme assay

For routine assays of UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine, the following system was developed: 50 μ l portions of homogenate were incubated in 13 ml stoppered centrifuge tubes with 1 mM-5-hydroxytryptamine, 6 mM-UDP-glucuronic acid and 0.6 M-triethanolamine/HCl buffer, pH 7.7, in a total volume of 250 μ l. In control tubes the UDP-glucuronic acid was added after incubation. Other additions were made as shown in the text. Incubations were terminated after 20 min by adding 5 ml of cold acetone to each tube. Free 5-hydroxytryptamine remains in solution, whereas the 5-hydroxytryptamine glucuronide is precipitated along with the triethanolamine/HCl. After shaking, the tubes were centrifuged for 10 min at 1200g, the supernatants decanted, and the tubes left inverted for 2 min to drain. Each precipitate was resuspended in a further 5 ml of acetone, centrifuged as before, and the resulting precipitate dissolved in 100 μ l of water. The solution was briefly heated to 100°C to denature any remaining protein, cooled, and treated with 1.0 ml of acidified *p*-dimethylaminobenzaldehyde (10%, w/v, in 7.5 M-HCl). After a further 10 min centrifuging the A_{550} of the resulting supernatant was measured. When added to the incubation mixture, digitonin, UDP-*N*-acetylglucosamine, diethylnitrosamine or Triton X-100 did not interfere with the assay system.

To prepare aqueous solutions of 5-hydroxytryptamine glucuronide for calibration, a 10% (w/v) mouse liver homogenate was incubated for 3 h with 2 mM-5-hydroxytryptamine, 8 mM-UDP-glucuronic acid and 0.2% (w/v) digitonin. After four washings with acetone, the final precipitates obtained from ten tubes were dissolved together in 1.0 ml of water.

Hydrolysis of 5-hydroxytryptamine glucuronide

The conjugate solution was incubated with 1 vol. of sodium acetate buffer (0.1 M, pH 5.5) and 1 vol. of

a β -glucuronidase preparation [a 10000g supernatant of a 20% (w/v) sonicate of female rat preputial gland in acetate buffer] for 6 h. Protein was denatured by heating to 95°C for 60 s, and removed by centrifugation. In control incubations the β -glucuronidase was added after incubation and in specifically inhibited samples; the acetate buffer was replaced by a 50 mM solution of D-glucaric acid 1,4-lactone (glucarolactone) in acetate buffer.

Other assays

UDP-glucuronosyltransferase activities towards *o*-aminophenol and bilirubin were assayed by the methods of Winsnes (1969) and Van Roy & Heirwegh (1968) respectively. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Optimal assay conditions

The HCl concentration of the *p*-dimethylaminobenzaldehyde reagent was critical for efficient formation of a complex with 5-hydroxytryptamine glucuronide. Although the molar absorption coefficient (ϵ_{550}) of the complex increased with increasing acid concentration to 7 M, acid concentrations above 8 M caused instability and rapid fading of colour. A *p*-dimethylaminobenzaldehyde concentration of at least 10% (w/v) was also required for efficient complex-formation, but higher concentrations slightly increased the absorbance of control samples. Preparations containing 7.5 M-HCl and 10% (w/v) *p*-dimethylaminobenzaldehyde when added to increasing concentrations of 5-hydroxytryptamine glucuronide formed a complex that obeyed Beer's law to A_{550} values of 0.6 and that remained stable for at least 20 min.

Identification of the reaction product

When mouse liver homogenates were assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine by the procedure described above, a reaction product was obtained that complexed with acidified *p*-dimethylaminobenzaldehyde to give a purple solution absorbing maximally at 550 nm. This product was identified as tryptamine 5-*O*- β -D-glucuronide from the following evidence. (i) The product's formation was dependent on 5-hydroxytryptamine, UDP-glucuronic acid and homogenate being present in the incubation mixture. (ii) Chromatography of the acetone-washed reaction product revealed (after spraying with Ehrlich's reagent) a single, purple, indole-positive spot with R_F 0.13. (iii) When portions of an aqueous solution of the acetone-washed

reaction products were incubated with β -glucuronidase, a compound was formed that, on subsequent chromatography, gave a blue, indole-positive spot with R_F 0.5. Non-conjugated 5-hydroxytryptamine had an identical R_F value and colour on chromatography under these conditions. (iv) Incubation in the absence of β -glucuronidase or with the addition of its specific inhibitor, glucarolactone, inhibited this conversion, thereby showing the reaction product to be a β -D-glucuronide. Blue rather than purple indole-*p*-dimethylaminobenzaldehyde complexes are formed with 5-hydroxyindoles (see Jepson, 1969). The purple colour of this glucuronide would therefore imply that the glucuronic acid is attached through O-5 on the indole ring.

Efficiency of substrate removal and product recovery

The double acetone-precipitation stage removed over 99% of the substrate present in control tubes (calculated from the differences in absorbance between control tubes without 5-hydroxytryptamine and those without added UDP-glucuronic acid). When added after incubation, however, UDP-glucuronic acid caused a small increase in control A_{550} values, thereby decreasing the apparent efficiency of 5-hydroxytryptamine removal to 98–99%. This increase of control values could occur in the absence of active enzyme. For this reason UDP-glucuronic acid was added to control tubes immediately after incubation in all assays.

The percentage recovery of glucuronide in this assay was determined by comparing the resulting A_{550} values of samples of a solution of 5-hydroxytryptamine glucuronide, added to control incubation tubes before incubation, with those of corresponding samples added to the final acetone precipitates from other control tubes immediately before the addition of the acidified *p*-dimethylaminobenzaldehyde. The percentage recoveries obtained ranged from 95 to 98%.

Calibration

As no purified 5-hydroxytryptamine glucuronide was commercially available, the conjugate's ϵ_{550} value was determined indirectly from that of 5-hydroxytryptamine, when both were complexed with *p*-dimethylaminobenzaldehyde, by the following procedure. A solution of the glucuronide, prepared as in the Materials and Methods section, was divided equally. Portion (1) was completely hydrolysed with β -glucuronidase, whereas portion (2) was incubated as an unhydrolysed control (complete, or lack of, hydrolysis being confirmed by chromatographing samples of the resulting deproteinized solutions). The resulting solutions (1) and (2) thus contained equimolar amounts of unconjugated and conjugated

5-hydroxytryptamine respectively. Serial dilutions of both solutions and a 5-hydroxytryptamine solution of known concentration were then complexed with acidified *p*-dimethylaminobenzaldehyde and their A_{550} values determined. The 5-hydroxytryptamine concentration in solution (1) and hence the glucuronide concentration in solution (2) could therefore be calculated from the ϵ_{550} value for the 5-hydroxytryptamine-*p*-dimethylaminobenzaldehyde complex. The ϵ_{550} values for the *p*-dimethylaminobenzaldehyde complexes of 5-hydroxytryptamine and 5-hydroxytryptamine glucuronide were found to be 1.10×10^4 and 1.55×10^4 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ respectively.

Optimal incubation conditions

Mouse liver UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine required relatively high (6mM) UDP-glucuronic acid concentrations for maximum activity in both native and digitonin-activated homogenates. Enzyme activity was optimal at a buffer pH of 7.7. Under these conditions reaction rates were linear for up to 40min with 2.5–5.0% (w/v) mouse liver homogenates. The assay could be used with very low protein concentrations. For example, non-activated 0.5% (w/v) homogenates of female rat liver gave, after a 30min incubation, A_{550} values of 0.040 ± 0.007 above controls. The creatinine sulphate component of the added substrate complex did not appear to interfere with enzyme activity, for the substrate could be replaced by 5-hydroxytryptamine oxalate without affecting enzyme activity. No apparent hydrolysis of product by β -glucuronidase occurred during incubation, for the addition of glucarolactone (up to 5mM) did not stimulate apparent conjugation.

Effects of perturbants

Membrane-perturbation procedures are known to activate UDP-glucuronosyltransferase activity towards other substrates (Lueders & Kuff, 1967; Winsnes, 1969; Burchell *et al.*, 1975). These procedures markedly stimulated mouse liver UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine. Excess perturbation in each case inactivated the enzyme. Because the degree of perturbation required to stimulate UDP-glucuronosyltransferase maximally can vary, the maximum stimulation of activity obtained by a given perturbation procedure was therefore determined by adding different concentrations of detergent (or preincubating or sonicating for different lengths of time) over the appropriate ranges and recording the maximum activity obtained. The different procedures clearly differed in their ability to stimulate UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine. Digitonin was the most efficient activator and could

stimulate activity 7–12-fold. Sonication and Triton X-100 were less efficient, stimulating activity less than 4-fold, whereas preincubation stimulated activity 6-fold. The addition of Triton X-100 to incubations of either sonicated or preincubated liver (samples from each sonication or preincubation time were assayed over the range of Triton X-100 concentrations) failed to stimulate activity more than either of the latter procedures did alone; these procedures were thus not additive.

UDP-*N*-acetylglucosamine

UDP-*N*-acetylglucosamine, an activator of UDP-glucuronosyltransferase activity towards many substrates (Pogell & Leloir, 1961; Winsnes, 1969), has been suggested as an effector in the endogenous control of the enzyme (Vessey *et al.*, 1973; Berry & Hallinan, 1976). The enzyme's activity towards 5-hydroxytryptamine was stimulated by UDP-*N*-acetylglucosamine, the optimum concentration being 3–4 mM. The degree of activation corresponded to that produced by Triton X-100 or sonication rather than to that produced by digitonin.

Diethylnitrosamine

Diethylnitrosamine has been shown to be a potent activator of rat liver UDP-glucuronosyltransferase activity towards xenobiotics such as *o*-aminophenol, *o*-aminobenzoate or 4-methylumbelliferone (Stevenson *et al.*, 1968; Winsnes, 1969; Zakim *et al.*, 1973), but a less effective activator of the mouse liver enzyme (see Dutton & Burchell, 1977). The effect of this nitrosamine on rat liver UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine was therefore investigated. No stimulation of this activity occurred when diethylnitrosamine was added over a concentrations of 5–25 mM. Activity towards *o*-aminophenol in the same homogenates could be stimulated 5–10-fold under these conditions.

Tissue distribution

Airaksinen *et al.* (1965) reported UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine to be present in homogenates of rat liver, kidney and duodenum, the latter tissue being the most active on a weight basis; activated activities were not measured, however. To compare activities between these and other tissues in the mouse, homogenates of the various tissues were assayed over a range of digitonin concentrations. As shown in Table 1, both duodenum and lung contain native UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine at similar values to that in liver when expressed per mg of protein. Lung, duodenum and kidney all contained less latent activity than liver, however.

Homogenates of mouse brain (20%, w/v) were also assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine over a range of digitonin concentrations, but no activity (limit of sensitivity 1 nmol/h per mg of protein) was detected.

Distribution between species and strain

5-Hydroxytryptamine glucuronide has been isolated from the bile of perfused Gunn rat liver (Tyce *et al.*, 1968b). It was therefore decided to determine whether hepatic UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine differed, in amount or activation characteristics, between Wistar and Gunn rats.

Liver homogenates from female homozygous Gunn rats or female Wistar rats were therefore assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine and bilirubin. As shown in Table 2, activity towards 5-hydroxytryptamine in Gunn rat liver homogenates was one-third of that in the Wistar; the effective absence of activity towards bilirubin confirmed the Gunn rats to be homozygotes (see Schmid *et al.*, 1958). To determine whether this decrease in enzyme activity was due to changes in

Table 1. UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine in homogenates of different mouse tissues. Homogenates of the various tissues were assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine over a range (0–0.2%, w/v) of digitonin concentrations, and the native (unactivated) and maximally activated (the highest activity obtained at any digitonin concentration for each sample) activities were recorded. Enzyme activity is expressed as nmol of 5-hydroxytryptamine glucuronide formed/h per mg of protein and is given for each tissue as the mean $\pm$ S.E.M. of a number (in parentheses) of experiments.

Tissue	Homogenate concn. (%, w/v)	UDP-glucuronosyltransferase activity		Activation (%)
		Native	Maximally activated	
Liver	3	26.3 $\pm$ 6.1 (6)	232.0 $\pm$ 14.0 (6)	881
Lung	10	22.6 $\pm$ 3.1 (6)	42.2 $\pm$ 2.9 (6)	187
Duodenum	10	27.7 $\pm$ 2.7 (6)	43.3 $\pm$ 2.5 (6)	156
Kidney	5	15.6 $\pm$ 5.5 (6)	33.4 $\pm$ 6.0 (6)	213
Brain	20	<1.0 (4)	<1.0 (4)	—

Table 2. *Typical UDP-glucuronosyltransferase activities in homogenates of liver from various species*

Homogenates (5%, w/v) of liver from the various species were assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine and bilirubin with or without the presence of digitonin in the incubation mixture. Activities are expressed in nmol/h per mg of protein and are given for each species as the mean and range from two animals. In cases where no activity was detected the limit of sensitivity of the assay is given in parentheses.

Species	Activation	UDP-glucuronosyltransferase activity:	
		towards bilirubin	towards 5-hydroxytryptamine
Mouse	Native homogenate	1.0 ± 0.1	23 ± 3
	+0.2% digitonin	7.9 ± 1.0	217 ± 5
Wistar rat	Native homogenate	0.5 ± 0.2	26.8 ± 0.5
	+0.2% digitonin	7.1 ± 0.6	87 ± 1.7
Gunn rat	Native homogenate	(<0.1)	8.7 ± 1.7
	+0.2% digitonin	(<0.1)	18.1 ± 1.4
Cat	Native homogenate	0.6 ± 0.1	(<1.5)
	+0.2% digitonin	1.1 ± 0.2	(<1.5)

Fig. 1. *Effect of digitonin on UDP-glucuronosyltransferase activity in Wistar and Gunn rat livers*

Liver homogenates (5%, w/v) from either female homozygous Gunn rats or Wistar rats were assayed for UDP-glucuronosyltransferase activity towards bilirubin (right-hand scale) and 5-hydroxytryptamine (left-hand scale) over the range of digitonin concentrations shown. Δ and $\blacktriangle$ indicate activity towards 5-hydroxytryptamine in the Gunn and Wistar rats respectively, and $\square$ and $\blacksquare$ indicate activity towards bilirubin in the Gunn and Wistar rats respectively. Each point represents the mean activity for two animals.

activation characteristics, liver homogenates from both strains were assayed over a range of digitonin concentrations. Fig. 1 shows that activity towards 5-hydroxytryptamine in both Gunn and Wistar rat liver show similar activation profiles, the activities for the Gunn rat being still only one-third of Wistar

rat activities at maximum activation. No activity towards bilirubin was observed at any digitonin concentration used. Addition of diethylnitrosamine (5–25 mM) also failed to stimulate activity towards 5-hydroxytryptamine in the Gunn rat liver homogenates.

As the cat family is known to be deficient in certain UDP-glucuronosyltransferase activities (see Millburn, 1974), cat liver homogenates were also assayed for UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine and bilirubin. As shown in Table 2, no activity towards 5-hydroxytryptamine could be detected, although activity towards bilirubin, stimutable by digitonin, was present. No activity towards either 5-hydroxytryptamine or *o*-aminophenol could be detected when cat liver homogenates were assayed over a range of digitonin concentrations to a maximum concentration of 0.2% (w/v).

Discussion

As the hepatic glucuronidation of 5-hydroxytryptamine is a normal process in some species (Tyce *et al.*, 1968a), this assay has the advantage of measuring a 'natural' UDP-glucuronosyltransferase activity. It has therefore been of use in the purification, and characterization of the differential development, of the enzyme (Leakey & Donald, 1976; Burchell, 1977; Wishart, 1978).

The activation characteristics of this UDP-glucuronosyltransferase activity are similar to those of other activities such as those towards *p*-nitrophenol or 4-methylumbelliferone in mouse liver (Winsnes, 1969). Lack of its stimulation by diethylnitrosamine is not surprising, as this activator can be highly substrate-specific (see Dutton & Burchell, 1977) and may not operate through membrane perturbation (Winsnes, 1969). The lower degree of latency of this UDP-glucuronosyltrans-

ferase activity in lung, duodenum and kidney compared with liver could reflect either low latency *in vivo* or more ready activation during homogenization; both explanations indicate tissue-specific differences in the membrane constraint of the enzyme.

The glucuronidating ability of Gunn rat liver is substrate-dependent. Activity towards bilirubin is completely absent, whereas activities towards morphine or testosterone are present at values near those of Wistar rats (for references see Dutton & Burchell, 1977). The condition can also affect the activation characteristics of some UDP-glucuronosyltransferase activities (Stevenson *et al.*, 1968; Zakim *et al.*, 1973). In Gunn rat liver, UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine is only one-third of that found in Wistar rats, but the activation characteristics of this activity of the enzyme (with respect to digitonin) remain unchanged.

In contrast with the Gunn rat, UDP-glucuronosyltransferase activity towards 5-hydroxytryptamine is completely absent from cat liver. Absence of this activity is significant, because although cat liver lacks activity towards many xenobiotic substrates, it contains activity towards all other endogenous substrates tested, including bilirubin, the steroids and thyroxine (see Myant, 1966; Williams, 1974; Millburn, 1974). The complete lack of this activity in cat liver is of interest for the overall metabolism of this amine, and the metabolic fate of 5-hydroxytryptamine in this species warrants further investigation.

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