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GLUCURONIDE AND SULPHATE CONJUGATION IN ISOLATED LIVER CELLS FROM CONTROL AND PHENOBARBITAL- OR PCB-TREATED RATS

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Abstract—The conjugation of 4-methylumbelliferone, *p*-nitrophenol and *o*-aminophenol in isolated rat liver cells was studied. It was found that for maximum sulphate conjugation to take place a very high sulphate concentration (50 mM) was needed, indicating that the rate limiting step may be the formation of 'active sulphate'. Under these conditions only a slight increase in the glucuronide/sulphate ratio with increasing substrate concentration was seen. Inducers of drug metabolism, PCB and phenobarbital, increased only glucuronidation. PCB was also found to cause qualitative changes in the metabolism of 4-methylumbelliferone.

Before being excreted from the body, many compounds, both exogenous and endogenous, are conjugated. Glucuronidation is quantitatively the most important conjugation reaction and has been studied extensively [1, 2]. However, many of the compounds that are glucuronidated are also sulphated [3, 4], and thus these two reactions compete for the same substrates. The affinity of the enzymes for the substrate, the polarity of the substrate and the availability of cofactors are among the factors that influence the relative amounts of glucuronidation and sulphation.

The enzyme catalysing glucuronidation, UDP-glucuronosyltransferase (UDPGT, EC 2.4.1.17) is a microsomal enzyme [1], whereas the sulphotransferases (ST, EC 2.8.2) are soluble enzymes [4]. Most of the studies on UDPGT have been done with microsomes, which eliminates the contribution of sulphation. In the present work the relationship between sulphation and glucuronidation was studied under physiological conditions, in intact liver cells. Cells were isolated from control, PCB-and PB-treated rats.

METHODS

Rats. Male Wistar rats, weighing 250–350 g, were used. They were fed on a standard laboratory diet. Rats treated with polychlorinated biphenyl (PCB) received an i.p. injection of Clophen A-50 (54 per cent w/w chlorinated biphenyl) dissolved in corn oil at 20 mg/ml (100 mg/kg body wt); control rats received corn oil only. The rats were used 2–4 weeks after the injection [5]. Phenobarbital (PB)-treated rats were given four daily i.p. injections of 80 mg/kg body wt [6] and were used the day after the last injection.

Isolation of cells. Liver cells were isolated essentially according to the method of Seglen [7]. The liver was perfused with a Ca^{2+} -free buffer followed by 0.05 per cent collagenase in a Ca^{2+} containing buffer. When the liver started to disintegrate the cells were freed with gentle rubbing and incubated for an additional 10 min at 37°. The cell suspension was filtered through gauze and centrifuged at 50 *g*. The cells were washed three times with a Hepes buffer containing 69 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.64 mM $MgCl_2$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4 and 30 mM Hepes at pH 7.4. This same buffer was also used for all incubations. The viability of the cells was 80–90 per cent, as judged by the exclusion of erythrocin B.

Conjugation of 4-methylumbelliferone. The reaction mixture contained (in a total volume of 5 ml) 2×10^5 cells/ml, 25–150 μM 4-methylumbelliferone (MU) and 50 mM sulphate (K_2SO_4). The incubation was carried out for 10–30 min at 37° with gentle shaking and was stopped by heating at 100°. After centrifugation 1 ml of the reaction mixture was passed through a 0.5×11 cm column of Dowex AG 50 W – X 8 (100/200 mesh) as described by Van Kempen and Jansen [8]. The column retained all of the MU with full recovery of 4-methylumbelliferyl-β-D-glucuronide (MUG) and 4-methylumbelliferyl sulphate (MUS) in the eluate (confirmed with standards). Aliquots of the eluate were treated with either 100 units of β-glucuronidase or 0.2 mg of arylsulphatase (in the presence of 10 mM saccharolactone) in a total volume of 2 ml 0.01 N Na-acetate buffer, pH 5.0, for 2 hr at 37°. The liberated MU was determined after the addition of 1 ml of 1.6 M glycine–NaOH buffer, pH 10,35, by measuring the fluorescence in an Aminco spectrofluorometer with an excitation wavelength of 370 nm and an emission wavelength of 450 nm. MUS was also hydrolysed with 0.4 N HCl for 30 min at 80° [8], and measured as above. Pure MUG and MUS were also used to confirm that there was no hydrolysis of MUS in the MUG determination and vice versa. Blanks included incubations without cells or without substrate, or incubations without subsequent hydrolysis. The activity was expressed as nmoles/min/10^6 cells.

Conjugation of p-nitrophenol. The conjugation of *p*-nitrophenol (PNP) was determined by the radioactive method of Marniemi and Hänninen [9], with some modifications. Incubations were carried out for 15–30 min at 37° in a total volume of 5 ml with 10^6

cells/ml, 50 mM K_2SO_4 and 25–200 μM PNP (100 or 200 nCi of [2, 6-^{14}C]-PNP). The reaction was stopped by heating, the precipitate removed by centrifugation and 1-ml samples were hydrolysed with β-glucuronidase or arylsulphatase as described above for MU conjugates. To the hydrolysed samples and one non-hydrolysed sample was added 0.1 ml of 30 per cent trichloroacetic acid and 1 ml of 1 M Tris-maleate buffer, pH 6.0. The samples were extracted with diethylether for removal of free PNP and 1 ml taken for the determination of radioactivity. After addition of 3 ml of Lumagel, the ^{14}C was counted with a Packard Tri-Carb scintillation counter. The non-hydrolysed sample gives the total conjugation, the arylsulphatase-hydrolysed sample the glucuronide conjugation and the β-glucuronidase-hydrolysed sample the sulphate conjugation. The total amount of conjugation was also determined by measuring the decrease in the absorbance at 410 nm after addition of NaOH. The activity was expressed as nmoles/min/10^6 cells.

Glucuronidation of o-aminophenol. The glucuronidation of *o*-aminophenol (OAP) was determined according to the method of Hartiala and Rontu [10] by measuring the appearance of diazotizable amine. Cells at 10^6 cells/ml were incubated at 37° with shaking for 15–30 min in a total volume of 5 ml in the presence of 50 mM K_2SO_4 and 110–385 μM OAP. The reaction was stopped by heating and the supernatant fraction analysed for diazotizable amine after the addition of 1M TCA–1M phosporic acid, pH 2.1, with *o*-aminophenylglucuronide (OAPG) as standard. The activity was expressed as nmoles/min/10^6 cells.

Statistical analysis. The results are expressed as means ± S.E. and analysed with the Wilcoxon matched-pairs signed-ranks test.

Reagents. Collagenase, type IV, deoxyribonuclease, type I, β-glucuronidase from bovine liver, type B-1, arylsulphatase and 4-methylumbelliferyl-β-D-glucuronide were from Sigma Chemical Co (St. Louis, MO, U.S.A.), 4-methylumbelliferylsulphate and *o*-aminophenylglucuronide from Koch-Light (Colnbrook, Bucks, U.K.), 4-methylumbelliferone and *p*-nitrophenol from Merck (Darmstadt, F.R.G.), *O*-aminophenol from Fluka (Buchs, Switzerland), sacharolactone from Pfizer (Folkestone, Kent, U.K.), Clophen A-50 from Bayer A.G. (Leverkusen, F.R.G.), [2, 6-^{14}C]-*p*-nitrophenol (16.7 and 38.8 mCi/mmole) from ICN (Irvine, CA, U.S.A.) and Lumagel from Lumac Systems (Basel, Switzerland). The other chemicals were reagent grade.

RESULTS

MU conjugation

In preliminary experiments the effect of sulphate concentration on the conjugation of MU was studied using high (100 or 150 μM) MU concentrations. It turned out that the sulphate concentration of the buffer (0.7 mM) was not enough to support maximum sulphation, but that a rather high concentration was needed. Thus 50 mM sulphate was used for the incubations, because it did not inhibit glucuronide formation, but gave optimum sulphation. Although this seems a very high concentration, even raising the sulphate concentration to 100 mM did not have inhibitory effects on either glucuronidation or sulphation, indicating that there were no adverse effects on the cells in this respect.

Under these conditions the glucuronide and sulphate conjugation reactions were linear for at least 40 min with 10^5 cells/ml and proportional to cell concentration up to 5×10^6 cells/ml for 30 min. However, when the formed MUS was determined using arylsulphatase hydrolysis the amount of MU liberated was not proportional to the amount of MUS formed. As the amount of reaction mixture in the hydrolysis incubation increased, the amount of liberated MU levelled off. This was not seen in HCl hydrolysis, and thus either HCl hydrolysis or both were used in subsequent experiments.

Conjugation patterns at various MU concentrations. The relative affinity and capacity of the liver cells for sulphation and glucuronidation was studied by incubating the cells in the presence of 50 mM sulphate at MU concentrations from 25 to 150 μM. As shown in Table 1, the amount of MUS formed remained relatively constant over this range, indicating that the sulphotransferase is saturated at low concentrations. The amount of MUG, on the other hand, showed a slight increase with increasing substrate concentration and was saturated at about 125 μM. Thus the MUG/MUS ratio was 0.65 at 25 μM MU and 0.90 at 150 μM—a significant increase ($P < 0.001$). However, if the MUG/MUS ratio was determined at the low sulphate concentration (0.7 mM) it increased much more with increasing MU concentration, as shown in the figure. This was

Table 1. Formation of MUS and MUG at different substrate concentrations in hepatocytes from control rats*

Substrate concentration† (μM MU)	n	nmoles/min/10^6 cells		MUG/MUS ratio
		MUS	MUG	
25	14	0.62 ± 0.07	0.43 ± 0.08	0.65 ± 0.08
50	8	0.69 ± 0.07	0.58 ± 0.09	0.85 ± 0.12
75	9	0.63 ± 0.08	0.50 ± 0.11	0.75 ± 0.10
100	7	0.69 ± 0.07	0.54 ± 0.11	0.79 ± 0.13
150	11	0.66 ± 0.06	0.62 ± 0.08	0.90 ± 0.09‡

* The values are given as means ± S.E.
† The concentration of K_2SO_4 was 50 mM.
‡ The MUG/MUS ratio is significantly higher ($P < 0.001$) at 150 μM MU than at 25 μM.

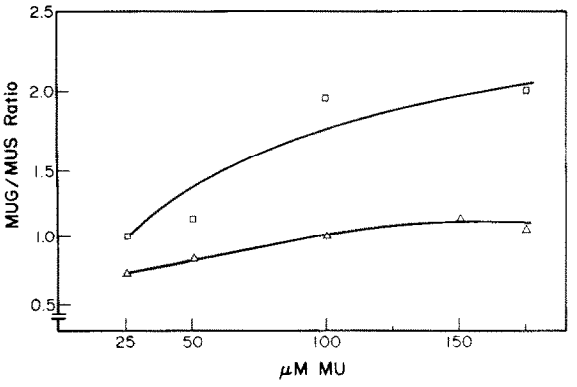

Fig. 1. The MUG/MUS ratio at different concentrations of MU in the presence of low (0.7 mM, □) or high (50 mM, △) K_2SO_4 concentrations. Isolated hepatocytes from control rats were incubated at 2×10^5 cells/ml and the formed conjugates determined as described in Methods.

due to a lower sulphation rather than increased glucuronidation and was more prominent at higher MU concentrations.

Effect of PB treatment on the conjugation of MU. Table 2 presents the results with liver cells from PB-treated rats. PB treatment increased the glucuronidation by 36 per cent (at 150 μM MU), but had little effect on the formation of MUS. Thus the MUG/MUS ratio was much higher with PB cells, as can be seen at all substrate concentrations.

Effect of PCB treatment. Table 3 shows the effect of PCB treatment on the glucuronidation of MU. There was an increase in the amount of MUG formed at all substrate concentrations, but the difference was fairly small and there also was more variability in the results than with control cells. At saturating concentrations of MU (150 μM) there is a 39 per cent increase as compared to control cells (the difference is statistically significant, $P < 0.05$). The quantitation of MUS was not possible since the cells formed some highly fluorescent compound(s) that were hydrolysed by arylsulphatase and HCl. The unhydrolysed 'blank' samples gave higher fluorescence than the hydrolysed samples.

The unknown compound that was formed by the PCB-treated rat hepatocytes seems to be a sulphate conjugate. It was hydrolysed under the same conditions as the regular MUS, i.e. with sulphatase and with acid, but was not hydrolysed by β-glucuronidase. In contrast to MUS, its fluorescence decreased upon hydrolysis. The formation of this metabolite was dependent on the presence of sulphate, MU and cells, and its formation was proportional to both time and cell concentration. Its formation was saturated at about the same substrate concentrations as that of MUS. In thin layer chromatography (with ethyl acetate–methanol–water as solvent) the product moved with about the same R_f value as MUS. When the incubation was carried out in the presence of $K_2{}^{35}SO_4$ the fluorescence and radioactivity coincided on t.l.c.

Table 3. Effect of PCB treatment of rats on the glucuronidation of MU in isolated liver cells*

Substrate concentration (μM MU)†	n	nmoles/min/10^6 cells	
		Control	PCB
25	8	0.42 ± 0.07	0.62 ± 0.06‡
50	6	0.56 ± 0.09	0.82 ± 0.10
75	4	0.41 ± 0.17	0.73 ± 0.24
100	5	0.57 ± 0.08	1.00 ± 0.21
150	6	0.53 ± 0.10	0.87 ± 0.21

* The values are given as means ± S.E.
† The concentration of K_2SO_4 was 50 mM.
‡ The increase is significant ($P < 0.05$) only at higher MU concentrations.

Conjugation of p-*nitrophenol*

Conjugation of PNP at various substrate concentrations. As with MU, the relative amount of sulphation and glucuronidation was dependent on the concentration of PNP (Table 4). The amount of *p*-nitrophenol sulphate (PNPS) did not increase when the substrate concentration was increased from 25 to 100 μM, whereas the amount of glucuronide increased significantly ($P < 0.005$). This gave an increase in the PNPG/PNPS ratio from 0.78 to 1.20 (statistically significant, $P < 0.005$). When the substrate concentration was 150 μM or more, there often was a considerable decrease in the conjugation, indicating a substrate inhibition or toxic effect on the cells (shown for PCB cells in Table 4). The sum of PNPG and PNPS was slightly lower than the total conjugation measured either with the radioactive method or spectrophotometrically (latter not shown, but gave similar results to the former).

Effect of PCB treatment on PNP conjugation. Table 4 also shows the effect of PCB treatment on the conjugation of PNP. The rate of sulphation is about the same as in control cells, but the glucuronidation is significantly higher (at 100 μM $P < 0.05$) and thus the PNPG/PNPS ratio is almost double in the cells from PCB-treated rats. This effect is seen at all substrate concentrations.

Table 2. Effect of phenobarbital treatment of rats on the conjugation of MU in isolated liver cells*

Substrate concentration (μM MU)†	n	MUG (nmoles/min/10^6cells)		MUS (nmoles/min/10^6 cells)		MUG/MUS	
		Control	PB	Control	PB	Control	PB
25	4	0.36 ± 0.17	0.65 ± 0.32	0.59 ± 0.21	0.65 ± 0.32	0.52 ± 0.12	1.06 ± 0.30
75	3	0.54 ± 0.15	0.83 ± 0.40	0.60 ± 0.16	0.43 ± 0.11	0.89 ± 0.04	1.75 ± 0.46
150	4	0.74 ± 0.07	1.01 ± 0.28	0.73 ± 0.05	0.68 ± 0.09	1.06 ± 0.15	1.58 ± 0.44

* The values are given as means ± S.E.
† The concentration of K_2SO_4 was 50 mM.

Table 4. Formation of PNPG and PNPS in isolated liver cells from control and PCB-treated rats at various concentrations of *p*-nitrophenol*

Substrate concentration (μM PNP)†	n	nmoles conjugate formed/min/10^6 cells			
		Total	PNPG	PNPS	PNPG/PNPS
Control cells					
25	8	0.82 ± 0.05	0.33 ± 0.02	0.46 ± 0.04	0.78 ± 0.09
50	3	1.26 ± 0.29	0.57 ± 0.16	0.57 ± 0.09	0.86 ± 0.16
100	8	1.20 ± 0.21	0.64 ± 0.13	0.38 ± 0.04	1.20 ± 0.21‡
PCB-treated cells					
25	8	0.86 ± 0.01	0.47 ± 0.04	0.37 ± 0.02	1.35 ± 0.16
50	3	1.45 ± 0.14	0.84 ± 0.07	0.42 ± 0.08	1.41 ± 0.27
100	8	1.55 ± 0.17§	0.74 ± 0.13§	0.45 ± 0.04	2.20 ± 0.31
200	3	0.51 ± 0.13	0.19 ± 0.01	0.18 ± 0.06	1.26 ± 0.30

* The results are given as means ± S.E.
† The concentration of K_2SO_4 was 50 mM.
‡ The ratio is significantly higher at 100 μM PNP than at 25 μM both in control and PCB-treated rats ($P < 0.005$).
§ PCB increases the total metabolism ($P < 0.025$) and glucuronide conjugation ($P < 0.05$).

OAP conjugation

Table 5 shows the glucuronidation of OAP in control and liver cells from PCB-treated rats. Here, adding sulphate seems to decrease the glucuronidation slightly, but sulphation was not measured. PCB causes an increase in the glucuronidation; the effect is seen at all substrate concentrations, and is more marked than with either MU or PNP as substrate.

DISCUSSION

The sulphation of phenolic compounds (both endogenous and exogenous) has been much less studied than the glucuronidation, even though the ability to form sulphate esters is widespread in nature [4]. Sulphation has been considered to be of minor importance due to a limited capacity. Many investigations, both *in vivo* and *in vitro* [11–15], indicate that glucuronidation becomes more important as the amount of substrate increases, but the effect varies with the drug and the conditions. There are also indications that the limiting factor may not be the sulphotransferase reaction *per se*, but rather that the amount of inorganic sulphate may be of importance. Both glucuronidation and sulphation need cofactors that are synthesized in energy requiring reactions, and thus factors other than the affinity and capacity of the enzyme must influence the conjugation reactions. Wiebkin *et al.* [16] studied the effect of metabolic inhibitors on the conjugation reactions and found that together with decreasing the level of ATP they also decreased the amount of conjugation. Sulphation was slightly more sensitive than glucuronidation to dinitrophenol.

The present study used very high concentrations of sulphate in the incubation mixture (50 mM). This is far above the physiological concentration, which is about 0.5–0.8 mM for rat [11], but not enough for optimum sulphation. The high sulphate concentration may have adverse effects on some cellular functions, but since both glucuronidation and sulphation were linear for at least 1 hr these reactions as well as the generation of cofactors were unaffected. The glucuronidation of both MU and PNP increased more than the sulphation with increasing substrate concentration, but the effect was less than 2-fold and also somewhat variable. These results differ quantitatively from those of Orrenius and coworkers [14, 15], who report a 10-fold increase in the PNPG/PNPS ratio and a 20-fold increase in the MUG/MUS ratio when the concentration of substrate increased from 25 to 200 μM in an incubation with isolated rat liver cells. They also found fairly large effects with other substrates. Besides the high sulphate concentration used in this study the differences may also be due to other differences in the incubation conditions and species of rat (Wistar vs

Table 5. *o*-Aminophenol glucuronidation in hepatocytes from control and PCB-treated rats*

Substrate concentration (μM OAP)	K_2SO_4 (mM)	n	(nmoles OAPG/min/10^6 cells)		
			Control	n	PCB
110	0.7	5	0.81 ± 0.08	3	1.92 ± 0.54†
110	50	4	0.72 ± 0.08	2	1.49 ± 0.45
220	0.7	8	1.12 ± 0.10	5	3.16 ± 0.55
220	50	8	1.23 ± 0.13	5	2.92 ± 0.49
385	0.7	6	1.29 ± 0.21	4	2.86 ± 0.60
385	50	4	1.08 ± 0.19	4	2.78 ± 0.73

* The results are given as means ± S.E.
† PCB treatment increases glucuronidation of OAP ($P < 0.05$).

Sprague–Dawley). However, Minck *et al.* [17] have measured the conjugation of PNP in isolated perfused rat liver over a substrate range of 25 to 100 μM. Both glucuronidation and sulphation increase and the PNPG/PNPS ratio goes from 0.64 to 1.54, which is closer to that found in this study. Hamada and Gessner [18], on the other hand, also using perfused rat liver, reported a constant glucuronidation rate at substrate concentrations from 25 to 500 μM, whereas the sulphation showed two phases, a faster one at low PNP and a slower one at higher PNP, giving PNPG/PNPS ratios of 2.7 and 4.3, respectively, also much lower than the ratios of Orrenius *et al.*

The rates found in the present study for sulphation of MU and PNP are close to those found by Orrenius *et al.* [15], but the glucuronidation rates are between the low and high substrate rates found by them. The glucuronidation rate found by Hamada and Gessner [18] with PNP as substrate converts to 0.22 nmoles/min/10^6 cells (using Seglen's [7] value of 19.4×10^7 hepatocytes/g liver) which is close to the one of this study, but their sulphation rate is slightly lower. The total conjugation of PNP was found to be slightly higher than the sum of PNPG and PNPS. This may be due to the formation of PNP-glucoside, which has also been shown in liver perfusions [17, 18].

The high sulphate concentration was found to decrease the glucuronide/sulphate ratio in this study. Büch *et al.* [12], in an *in vivo* experiment, found that the amount of sulphate has an effect on the conjugation pattern. They injected *N*-acetyl-*p*-aminophenol into rats and measured the excreted conjugates. When Na_2SO_4 was injected together with the drug, there was a significant increase in the amount of sulphated drug. Similarly, Mulder and Keulemans [19] found that the ratio between harmol glucuronide and harmol sulphate decreased from 9 to 2 when 1.3 mM sulphate was added to the perfusion medium in a liver perfusion. Without added sulphate the pool soon became exhausted. With *o*-aminophenol as substrate only the glucuronidation was measured. High sulphate was found to decrease the glucuronidation, as has been observed earlier in liver slices [20]. The effect is due to sulphate conjugation, and is not a direct effect of sulphate [20].

The effect of sulphate is somewhat difficult to interpret, as it may not be the sulphate conjugation that is affected directly, but the synthesis of active sulphate, 3′-phosphoadenosine-5′-phosphosulphate (PAPS) [4, 21]. This pool is apparently very small, and the synthesizing enzyme has a high K_m value for sulphate (1.3 mM) [4]. Thus the pool of PAPS may be depleted when the demand for conjugation is high, especially at low sulphate concentrations.

PB treatment caused an increase in the glucuronidation of MU when calculated on a cell number basis, as was also found by Andersson *et al.* [14], but there was no effect on the sulphation. Bock and Fröhling [22] also found an increase in the glucuronidation of *L*-naphthol in perfused rat liver with a slight decrease in sulphation (calculated per g liver). PB has also been shown by others [23, 24] to increase glucuronidation. Büch *et al.* [12] found an increase in the *in vivo* sulphation of *N*- acetyl-aminophenol by PB, if measured after injection of inorganic sulphate.

PCB treatment also increased glucuronidation in the present study with all three substrates, MU, PNP and OAP. The sulphation of PNP was not affected. There are few studies on the effect on sulphation, but Gessner and Hamada [18] report an increase in both the glucuronidation and sulphation of PNP in livers from 3-methylcholanthrene-treated rats. Andersson *et al.* [14] have found an increase in the conjugation of 2-napthol, but consider it to be due only to an increase in glucuronidation. The effect of PCB on the sulphation of MU in the present study is difficult to interpret because there are also qualitative changes. It seems likely that PCB, which is known to increase mixed function oxidases [5], causes hydroxylation of MU, and that this new hydroxyl group is then sulphated. Such a change in metabolic pattern has been observed with e.g. biphenyl—liver cells from control rats metabolize it mainly to 4-OH-biphenyl, but 3-methylcholanthrene pretreatment causes also 2- and 3-hydroxylation, and these groups are then conjugated [25].

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