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Studies of Mammalian Glucoside Conjugation

By T. GESSNER,* A. JACKNOWITZ and C. A. VOLLMER
Department of Biochemical Pharmacology,
State University of New York at Buffalo,
Buffalo, N.Y. 14214, *U.S.A.*

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The mammalian glucoside-conjugation pathway was studied by using *p*-nitrophenol as the model substrate and mouse liver microsomal preparations as the source of enzyme. The microsomal preparations supplemented with UDP-glucose glucosylated *p*-nitrophenol; *p*-nitrophenyl glucoside was identified by chromatography in six solvent systems. The unsolubilized glucosyltransferase of fresh microsomal preparations did not follow the usual Michaelis–Menten kinetics and was easily inhibited by many steroids. All the steroids tested inhibited glucosylation of *p*-nitrophenol to a greater degree than glucuronidation of *p*-nitrophenol when assayed in the same microsomal preparations. The steroids inhibited glucosylation with the following decreasing effectiveness: pregnan-3α-ol-20β-one (3α-hydroxypregnan-20-β-one) > oestradiol-17β 3-methyl ether > oestradiol-17β > oestriol > pregnane-3α,20β-diol > oestrone. Pregnan-3α-ol-20β-one, pregnane-3α,20β-diol and oestrone had negligible effect on glucuronidation.

Vertebrates are known to conjugate many exogenous and some endogenous phenolic, alcoholic, carboxylic, amino or mercapto compounds with glucuronic acid (Smith & Williams, 1966; Dutton, 1966*a*) by transfer of the acid moiety from UDP-glucuronic acid to the aglycones (Dutton, 1966*a*). In plants, bacteria, insects and molluscs, glucosyl derivatives are formed by an analogous transfer of glucose from UDP-glucose to aglycones (Yamaha & Cardini, 1960; Smith & Turbert, 1961; Gessner & Acara, 1968; Dutton, 1966*b*; Illing & Dutton, 1970). The two pathways of conjugation, i.e. glucuronide and glucoside formation, have been on occasions regarded as mutually exclusive (Dutton, 1966*b*) and glucosylation of exogenous compounds has been viewed as a detoxication mechanism characteristic of invertebrates and other lower organisms (Smith, 1968). Since we first reported a *p*-nitrophenol-glucosylating system in mouse liver microsomal fraction (Gessner & Vollmer, 1969) results have been accumulating indicating that glycosylation is a pathway of widespread occurrence in mammals and of considerable consequence, since such important endogenous compounds as oestrogens and bilirubin are found in the form of glucoside conjugates. Williamson *et al.* (1969) reported the finding of oestradiol-17β 17-glucopyranoside in the glucuronidase-hydrolysed urine of rabbits treated with large doses of oestrone benzoate. The urine of mice given *p*-nitrophenol also contained small quantities of *p*-nitrophenyl glucoside (Gessner & Hamada, 1970). Collins *et al.* (1970) reported that partially purified steroid glucosyltransferase from rat liver microsomal preparations was able to transfer glucose from UDP-glucose to oestradiol-17α 3-glucuronoside, oestradiol-17α, oestradiol-17β, oestrone and diethylstilboestrol. With regard to bilirubin conjugation, Kuenzle (1970*a*,*b*,*c*) found glucose conjugates of bilirubin in the form of disaccharide conjugates of bile pigments. Fevery *et al.* (1971) and Compernolle *et al.* (1971) found β-D-monoglucoside of bilirubin present as an ester conjugate, in substantial amounts in dog bile and also in lesser amounts in human post-obstructive bile. Heirwegh *et al.* (1971) and Wong (1971) reported on microsomal glucosylation of bilirubin.

In the present work we have used *p*-nitrophenol as a substrate to study the mammalian glucosylation pathway and the properties of the enzyme system involved, and its relationship to the glucuronide-conjugation pathway, especially with respect to the inhibitory action of steroids.

Materials and Methods

Chemicals

p-Nitrophenol, 'ultra pure' grade, was obtained from Mann Research (Schwarz–Mann, Orangeburg, N.Y., U.S.A.). UDP-D-[U-^{14}C]glucose of 98.7% radiochemical purity and UDP-D-[U-^{14}C]glucuronic acid of 99.1% radiochemical purity were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. *p*-Nitro[2,6-$^{14}C_2$]phenol of 99% radiochemical purity was from Tracer Laboratories (Waltham,

* Present address: Department of Experimental Therapeutics, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N.Y. 14203, U.S.A.

Mass., U.S.A.). The following chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.): UDP-glucose; UDP-glucuronic acid; oestrone (3-hydroxy-$\Delta^{1,3,5(10)}$-oestratrien-17-one); oestradiol-17β ($\Delta^{1,3,5(10)}$-oestratriene-3,17β-diol); oestradiol 3-methyl ether ($\Delta^{1,3,5(10)}$-oestratriene-3-17β-diol 3-methyl ether); oestriol ($\Delta^{1,3,5(10)}$-oestratriene-3,16α,17β-triol); pregnanediol (5β-pregnane-3α,20β-diol); 5β-pregnan-3α-ol-20-one (3α-hydroxy-5β-pregnan-20-one); Tris buffer; D-glucose; *p*-nitrophenyl β-D-glucoside; *p*-nitrophenyl β-D-glucuronide; *p*-nitrophenyl sulphate. All solvents and other chemicals were Fisher (Springfield, N.J., U.S.A.) certified reagents. Scintillation-grade 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Packard Instrument Co. (Downers Grove, Ill., U.S.A.). Crystalline bovine serum albumin was from Pierce Chemical Co. (Rockford, Ill., U.S.A.).

Enzyme preparations

Freshly excised livers of adult male Swiss Webster mice (body wt. 30–40g) were used for the preparations. All manipulations were carried out at 0–4°C. After excision of gall bladders, combined livers of three to five animals were rinsed in ice-cold 0.85% NaCl, blotted with Whatman filter paper and homogenized in 0.25M-sucrose or in 0.15M-KCl containing 1mM-EDTA, to give 10–20% (w/v) homogenates. Homogenization was carried out by two to four passes in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle at 1000rev./min. In the fractionation experiments the homogenates were filtered through gauze before centrifugation. For the study of glucose 6-phosphatase, glucosyltransferase and glucuronyltransferase activities, all particulate fractions were first separated from the soluble supernatant by centrifugation at $100000g_{av.}$ for 1h as suggested by Ricketts (1964). The particulate pellet was then resuspended in sucrose by homogenization as above for 1min at 1000rev./min and fractions were prepared by centrifuging as follows: nuclear fraction, $755g_{av.}$ for 10min; mitochondria, $5090g_{av.}$ for 10min; lysosomes, $12100g_{av.}$ for 10min; microsomal fraction, $105000g_{av.}$ for 60min. All the particulate fractions were washed by gently mashing the pellets by hand with a Teflon pestle and repeating the centrifugations. The supernatant was also centrifuged again at $105000g_{av.}$ for 1h to clear it of any remaining particles. Good-quality subcellular fractions were obtained, as ascertained by electron microscopy. In the experiments where $25300g_{av.}$ and $105000g_{av.}$ were needed the procedure reported by Leloir & Goldemberg (1960) was used. When studies called for a microsomal preparation only, the fraction was obtained by spinning the homogenate at $12100g_{av.}$ for 20min, discarding the pellet and spinning the supernatant at $105000g_{av.}$ for 60min. The microsomal pellet was washed twice as described above, and then resuspended by homogenization at 1000rev./min for 30s in a volume of 0.25M-sucrose equal to the wet weight of liver from which it originated. Protein content of enzyme preparations was determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as the standard.

Assays of glucosyltransferase and glucuronyltransferase

All transferase assays were performed on homogenates and subcellular fractions that had not been stored. Biosynthesized *p*-nitrophenyl glucoside or *p*-nitrophenyl glucuronide was detected and determined by measuring incorporation of ^{14}C either from *p*-nitro[^{14}C]phenol or from UDP-[^{14}C]glucose or from UDP-[^{14}C]glucuronic acid. A typical incubation mixture consisted of the following: enzyme preparation (equivalent to about 0.5g of liver/ml of mixture), 5mM-*p*-nitrophenol, 10mM-UDP-glucose or 5mM-UDP-glucuronic acid, label equivalent to 0.1–0.2μCi/ml of mixture, 0.2M-Tris–HCl buffer, pH6.8, unless otherwise indicated. Aerobic incubations were carried out at 37°C in a Dubnoff metabolic shaker for 10–60min as indicated. The reactions were stopped by addition of 3vol. of ethanol, and the samples were then immediately frozen on solid CO_2, sealed and stored at −20°C until used for analysis. The samples were thawed, centrifuged and the supernatants applied quantitatively (0.2ml volumes) to paper strips.

Chromatography and determination of metabolites

Descending chromatograms were developed on Whatman no. 1 or no. 4 paper in the following solvent systems: (A) benzene–butan-1-ol–pyridine–water (1:5:5:3, by vol.); (B) butan-1-ol–acetone–acetic acid–aq. 5% NH_3 (7:5:3:3, by vol.); (C) butan-1-ol–pyridine–water (14:3:3, by vol.); (D) propan-1-ol–aq. NH_3 (sp.gr. 0.880)–water (6:3:1, by vol.); (E) benzene–99.7% acetic acid–water (125:72:3, by vol.); (F) methanol–90.4% formic acid–water (16:3:1, by vol.).

Reference compounds were always chromatographed simultaneously with metabolite samples; moreover, samples of unlabelled reference compounds were applied on top of the metabolite sample spots for co-chromatography. All reference compounds, except for glucose, were detectable as quenching spots under u.v. light. Glucose was detected with the aid of an $AgNO_3$ spray (Block *et al.*, 1964).

Chromatograms were scanned with a Nuclear-Chicago 4Pi Radiochromatogram Scanner/Integrator/Printer assembly, which was used to locate the

peaks and to measure their radioactivity by comparison with standard amounts of precursor treated similarly. In kinetic experiments, the areas of chromatograms containing the metabolites under investigation were cut into bands (0.5cm × 2.52cm) and immersed in toluene scintillation fluid [1 litre of toluene containing 4.0g of 2,5-diphenyloxazole and 0.5g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (Gessner & Acara, 1968)]. Radioactivity was measured to a standard error of 5% with a Nuclear-Chicago Unilux scintillation counter, model 6850; the counting efficiency in this procedure was 40%.

Assay of glucose 6-phosphatase

The assay was carried out on enzyme preparations that were kept frozen at −20°C for up to 3 days; under these conditions of storage no appreciable loss in the enzyme activity was detectable. Glucose 6-phosphatase was determined by the standard method of Harper (1963) and phosphate by that of Fiske & SubbaRow (1929).

Results

p-Nitrophenyl glucoside as a metabolite of p-nitrophenol

Mouse liver fractions prepared in 0.15M-KCl were incubated with 5mM-UDP-glucose and 5mM-*p*-nitrophenol as substrates in 50mM-Tris-HCl buffer, pH7.1, containing 1mM-EDTA. A radioactive metabolite with the mobility of *p*-nitrophenyl glucoside in solvent (B) was detectable in incubations irrespective of which substrate was radioactively labelled. Moreover, in an experiment where liver homogenate was incubated in three different ways with the labelled precursors, namely, (i) unlabelled *p*-nitrophenol with UDP-[^{14}C]glucose, (ii) *p*-nitro[^{14}C]phenol with unlabelled UDP-glucose or (iii) *p*-nitro[^{14}C]phenol with UDP-[^{14}C]glucose, the metabolite peak at the R_F of *p*-nitrophenyl glucoside obtained from incubation (i) contained 980c.p.m., that from incubation (ii) 1946c.p.m. and that from incubation (iii) 2898c.p.m. The fact that the radioactivity of *p*-nitrophenyl glucoside labelled in both moieties was equal to the sum of the other two provided further proof of the formation of *p*-nitrophenyl glucoside. In other experiments, where liver homogenate, and fractions thereof, were incubated as before with UDP-[^{14}C]-glucose, comparable amounts of radioactive metabolite were found at the R_F of *p*-nitrophenyl glucoside in both solvent systems (A) and (B) (see Table 1), thus confirming the identity of the product.

The identity of the metabolite of *p*-nitrophenol from mouse liver incubations supplemented with UDP-[^{14}C]glucose was further confirmed by chromatography in six solvent systems (see Table 2). Extract of metabolite mixture, obtained from an incubation with mouse liver homogenate supplemented with UDP-[^{14}C]glucose, was streaked on a sheet of Whatman no. 1 paper and chromatographed in solvent (B). The radioactive band corresponding to the R_F of *p*-nitrophenyl glucoside was eluted with water. Portions of the eluate were chromatographed in three solvent systems, (A), (C) and (E), and in each case the metabolite behaved like *p*-nitrophenyl glucoside (Table 2). Moreover, the spot of the metabolite with the same R_F as the glucoside in solvent (C) was cut out and subjected to further chromatography (without elution) consecutively in solvents (D) and (E) (Gessner & Hamada, 1970). Therefore the metabolite that behaved like *p*-nitrophenyl glucoside in solvent (B) also migrated like the glucoside in solvents (C), (D) and (E), thus confirming its identity.

Table 1. *Incorporation of ^{14}C from UDP-[^{14}C]glucose into a metabolite with the mobility of p-nitrophenyl glucoside*

Mouse liver preparations in 0.15M-KCl were incubated with 5mM-*p*-nitrophenol and 5mM-UDP-[^{14}C]glucose at pH7.1 in 50mM-Tris-HCl buffer containing 1mM-EDTA. The metabolite was determined by radiochromatography as described in the Materials and Methods section.

Liver fraction	Time (min)	Conversion of UDP-glucose into metabolite with R_F of *p*-nitrophenyl glucoside (%)	
		Solvent (A)	Solvent (B)
Whole homogenate	30	1.9	2.1
	90	4.1	3.7
Particles (25300*g*)	30	1.3	1.5
	90	4.3	3.9
Supernatant (105000*g*)	30	0	0
	90	0	0
Particles (105000*g*)	30	4.4	4.0
	90	12.5	13.5

Table 2. *Comparison of R_F values of reference compounds and of the metabolite of UDP-[^{14}C]glucose*

Mouse liver homogenate prepared in 0.25M-sucrose was incubated with 5mM-*p*-nitrophenol and 5mM-UDP-[^{14}C]glucose in Tris–HCl buffer as described in Table 1. Extracts of metabolites were chromatographed on Whatman no. 1 paper in the solvent systems listed in the Materials and Methods section; the chromatograms were scanned as described in the Materials and Methods section. Metabolite mobility was always compared with the reference samples simultaneously chromatographed under the same conditions.

	R_F					
Solvent ... / Compound	(A)	(B)	(C)	(D)	(E)	(F)
UDP-glucose	0.03	0.07	0.01	0.10	0.10	0.29
Glucose	0.40	0.30	0.19	0.50	0.16	0.69
p-Nitrophenol	0.85	0.90	0.90	0.82	0.78	0.83
p-Nitrophenyl glucuronide	0.42	0.47	0.18	0.70	0.32	0.70
p-Nitrophenyl sulphate	0.71	0.65	0.50	0.85	0.20	0.68
p-Nitrophenyl glucoside	0.81	0.66	0.62	0.87	0.45	0.73
Metabolite	0.83	0.66	0.59	0.85	0.45	0.73

Table 3. *Subcellular distribution of p-nitrophenyl glucoside-synthesizing activity*

Mouse liver homogenates were prepared in 0.25M-sucrose containing 1mM-EDTA and were fractionated as described in the text. In this experiment concentrations of all the substrates, *p*-nitrophenol, UDP-[^{14}C]glucose and UDP-[^{14}C]glucuronic acid, were 5mM; incubations were carried out in 50mM-Tris–HCl buffer, pH7.1; microsomal incubations contained 14mg of protein/ml. Metabolites were chromatographed in solvent (B) and the chromatograms scanned as described in the Materials and Methods section. It was ascertained that control incubations, containing labelled UDP-glucose or UDP-glucuronic acid as precursors, did not yield any interfering metabolites with R_F values of *p*-nitrophenyl glucoside or *p*-nitrophenyl glucuronide. Moreover, in this and other experiments, there was no detectable formation of *p*-nitrophenyl glucoside in incubations that yielded *p*-nitrophenyl glucuronide and vice versa, thus showing that there was no detectable interconversion between *p*-nitrophenyl glucuronide and *p*-nitrophenyl glucoside.

	Synthesis (μmol/100mg of protein)				Glucose 6-phosphatase activity (μmol of P_i/mg of protein)	
	p-Nitrophenyl glucoside		*p*-Nitrophenyl glucuronide			
Liver fraction	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Whole homogenate	0.336	0.26	1.44	2.39	1.8	1.2
Nuclear (775*g*)	0.198	0.06	—	—	0.94	0.55
Mitochondrial (5090*g*)	0.013	0.012	—	—	0.65	0.21
Lysosomal (12100*g*)	0.222	0.18	—	—	4.31	3.05
Soluble (105000*g*)	0	0	—	—	0	0.03
Microsomal (105000*g*)	1.21	0.929	4.25	5.14	6.54	4.81

Subcellular location of p-nitrophenol UDP-glucosyltransferase

Since *p*-nitrophenyl glucoside-synthesizing activity partially sedimented at 25300$g_{av.}$ and at 105000$g_{av.}$ (Table 1), a more systematic study of the subcellular distribution was undertaken, together with measurement of the microsomal marker enzyme glucose 6-phosphatase. For this purpose all the particulate fractions were separated first according to the suggestion of Ricketts (1964), who found that glucose 6-phosphatase activity is progressively lost during preincubation of microsomal fraction with the soluble fraction. The fractions were prepared in 0.25M-sucrose containing 1mM-EDTA, and incubated with 5mM-UDP-[^{14}C]glucose and 5mM-*p*-nitrophenol, and the products measured as described above. For comparison, glucuronyltransferase activity, which is known to be microsomal, was also

measured with *p*-nitrophenol and UDP-[^{14}C]glucuronic acid as substrates. The highest glucosyltransferase activity was associated with the microsomal fraction (Table 3). In Expt. 1 the particulate fractions were washed only once, whereas in Expt. 2 they were washed twice. The additional washing seems to have resulted in somewhat better fractionation and also in a less-active microsomal preparation. The lysosomal fraction in both experiments appeared to have significant glucosylating activity, which is probably due to microsomal contamination. The high glucose 6-phosphatase content of this fraction is in part attributable to other lysosomal phosphatases.

Properties of the microsomal glucosyltransferase

To study the activity of the mouse liver microsomal system responsible for the glucosylation of *p*-nitrophenol at various pH values, the microsomal fractions, which were prepared as in the previous experiment, were incubated with substrates in the presence of 0.12M-Tris–HCl and Tris–maleate buffers (Fig. 1). The optimum pH of the reaction was close to pH 7, between 6.8 and 7.1.

All subsequent studies were done on microsomal fractions without separation of other subcellular particles, and radioactivity was measured by scintillation counting. The rate of *p*-nitrophenyl glucoside formation was linear for the first 30min and then slowly began to decline (Fig. 2). All subsequent experiments were done with 30min incubation times.

To study the kinetics of *p*-nitrophenyl glucoside synthesis, various concentrations of UDP-glucose were incubated with a constant concentration of *p*-nitrophenol and vice versa. The constant concentration of *p*-nitrophenol was 5mM and that of UDP-glucose 10mM. The conversion rate increased with increasing concentrations of UDP-glucose up to 10mM (Fig. 3). A Lineweaver–Burk plot of this experiment indicated that the reaction does not obey Michaelis–Menten kinetics (Fig. 4). This provided an explanation for the variations in the apparent K_m values in some of our earlier experiments, which were carried out within various but narrow concentration ranges of UDP-glucose. In such experiments the points appeared to be on a straight line, but the estimates of K_m ranged from 0.5 to 2.5mM. Thus, in the last experiment of Fig. 4, by choosing either a low (0.1–1mM) or a high (1–10mM) concentration range one could also plot apparent straight lines through four points each and obtain different apparent K_m values as before. The relationship between *p*-nitrophenol concentration and *p*-nitrophenyl glucoside synthesis also deviated from typical Michaelis–Menten kinetics, the curve being sigmoid (Fig. 5).

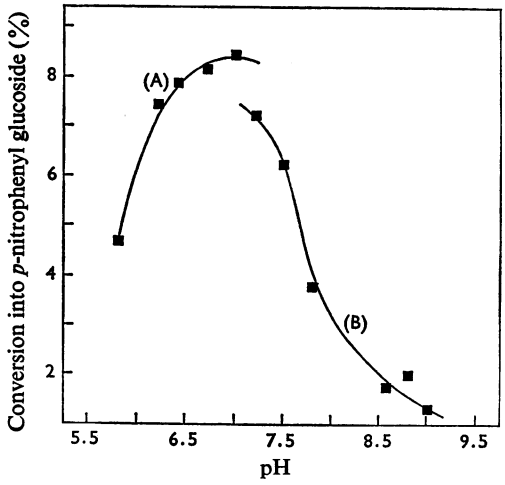

Fig. 1. *Effect of pH on mouse liver microsomal p-nitrophenol glucosyltransferase*

Activity was assayed (A) in 0.12M-Tris–maleate buffer, and (B) in 0.12M-Tris–HCl buffer. Incubations contained substrates *p*-nitrophenol and UDP-[^{14}C]-glucose (each 5mM) and were carried out as described in the Materials and Methods section.

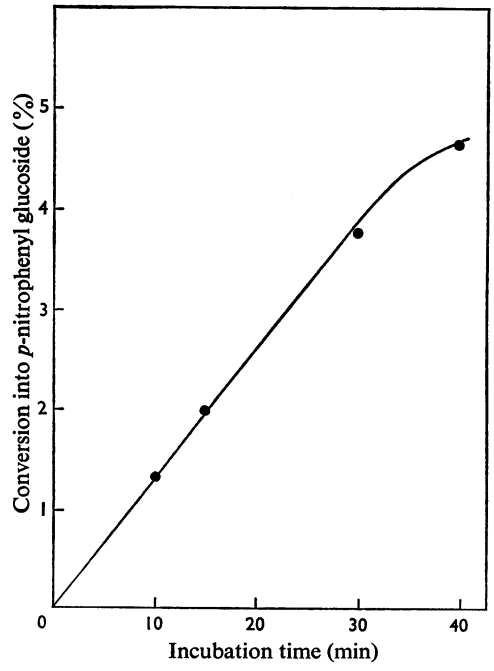

Fig. 2. *Time-course of microsomal glucosylation of p-nitrophenol*

Incubation conditions were as given in the Materials and Methods section.

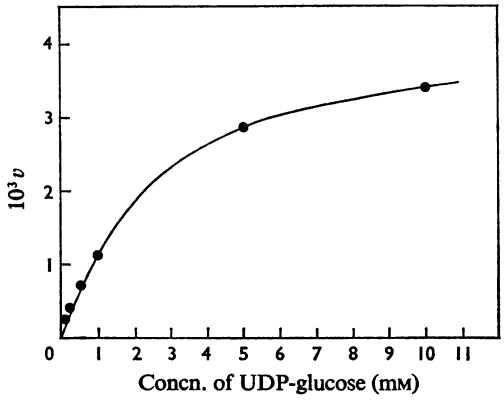

Fig. 3. *Effect of concentration of UDP-glucose on the rate of glucosylation of p-nitrophenol*

The incubations were carried out at 37°C as described in the Materials and Methods section, with 5mM-*p*-nitrophenol. Activity (v) is expressed in μmol of *p*-nitrophenyl glucoside formed/30min per mg of microsomal protein.

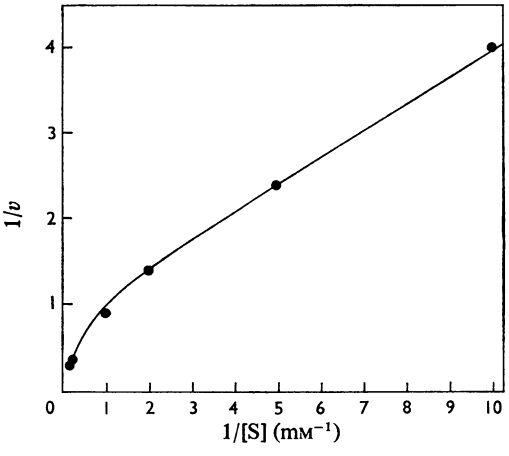

Fig. 4. *Double-reciprocal plot of activity against concentration of UDP-glucose*

Conditions of incubation were as given in Fig. 3. Activity (v) is expressed in nmol of *p*-nitrophenyl glucoside formed/30min per mg of microsomal protein.

Inhibitors of p-nitrophenyl glucoside synthesis

In view of the observation that rabbit liver microsomes contained a steroid-glucosylating system capable of transferring glucose from UDP-glucose to the

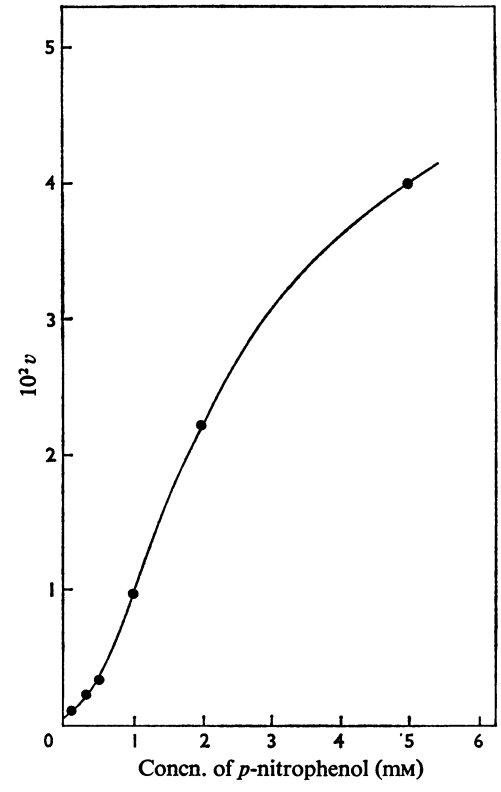

Fig. 5. *Effect of concentration of p-nitrophenol on the rate of synthesis of p-nitrophenyl glucoside*

The incubations were carried out at 37°C, as described in the Materials and Methods section, with 10mM-UDP-glucose. Activity (v) is expressed in μmol of *p*-nitrophenyl glucoside formed/30min per mg of microsomal protein.

17α-hydroxyl group of oestradiol-17α 3-glucuronoside as well as to the phenolic 3-hydroxyl group of oestrone and oestradiol (Collins *et al.*, 1970), it was decided to test whether the glucosylation that we observed with an exogenous phenol was also related to the metabolism of endogenous substrates, such as steroids and especially oestrogens with their phenolic hydroxyl group. To explore this we tested the inhibitory effect of the steroids on the glucosylation of *p*-nitrophenol and compared this with their effect on glucuronidation of *p*-nitrophenol assayed on the same enzyme preparations.

Microsomal preparations suspended in 0.25M-sucrose were incubated with *p*-nitrophenol and either UDP-[^{14}C]glucose or UDP-[^{14}C]glucuronic acid in Tris–HCl buffer, at pH 6.8 for glucosylation or pH 8.0 for glucuronidation (these being the pH values close

Table 4. *Inhibitors of microsomal glucosylation of p-nitrophenol: a comparison with glucuronidation*

Liver microsomal preparations suspended in 0.25M-sucrose and Tris–HCl buffer were incubated with *p*-nitrophenol and either UDP-[^{14}C]glucose of UDP-[^{14}C]glucuronic acid, in the presence and absence of a given inhibitor. Steroid inhibitors were added as propylene glycol solutions; control incubations contained propylene glycol alone. Incorporation of ^{14}C into *p*-nitrophenyl glucoside or *p*-nitrophenyl glucuronide was determined by scintillation assay as described in the Materials and Methods section. Steroid glucuronides were found to overlap with the peak of *p*-nitrophenyl glucuronide on chromatograms developed in solvent (B). The percentage of radioactivity due to steroid conjugate(s) was as follows: oestrone, 3%; oestradiol, 7.3%; oestradiol 3-methyl ether, 6.3%; oestriol, 8.3%; pregnane-3α,20β-diol, 0.5%; pregnan-3α-ol-20β-one, 0.3%.

Inhibitor	Inhibition (%)	
	Formation of *p*-nitrophenyl glucoside	Formation of *p*-nitrophenyl glucuronide
Oestrone (5mM)	27.8	3.9
Oestradiol-17β (5mM)	50.6	12.4
Oestradiol-17β 3-methyl ether (5mM)	63.7	43.7
Oestriol (5mM)	43.6	30.1
Pregnane-3α,20β-diol (5mM)	34.0	3.5
Pregnan-3α-ol-20β-one (5mM)	77.1	0.2
UDP-glucuronic acid (2mM)	65.1	—

to the optima). Steroids dissolved in propylene glycol were added to incubations to give 5mM solutions and control incubations contained propylene glycol alone. In separate experiments under the same experimental conditions propylene glycol decreased glucosylation by 7% and glucuronidation by 16%. Additional control incubations, which contained the steroids in propylene glycol but lacked *p*-nitrophenol, were also done to determine possible interference of steroid conjugates with the *p*-nitrophenyl glucoside or *p*-nitrophenyl glucuronide assay. With solvent system (B) no interference was observed at the R_F value of *p*-nitrophenyl glucoside, but steroid conjugates contributed 0.8–8.0% of radioactivity in the glucuronide R_F region and appropriate corrections were therefore applied.

Table 4 shows that all the steroids tested were more inhibitory to *p*-nitrophenol glucosylation than to its glucuronidation and that, contrary to expectations, steroids bearing phenolic 3-hydroxyl groups were less effective as inhibitors than those with alcoholic 3-hydroxyl groups, such as pregnan-3α-ol-20-one. Of the steroids tested the latter compound appeared to be the most potent inhibitor of glucosylation, but had no effect on glucuronidation. Pregnane-3α,20β-diol was an effective inhibitor of glucosylation but had very little effect on glucuronidation. This latter observation is in agreement with the report of Adlard & Lathe (1970*a*), who found pregnane-3α,20β-diol ineffective in inhibiting microsomal glucuronidation of bilirubin, in spite of earlier reports (Bevan *et al.*, 1965) on the effect of the steroid on bilirubin conjugation. Bilirubin has also been found to undergo microsomal glucosylation (Wong, 1971). Therefore, in the light of our results, it appears likely that the steroid exerts its inhibitory effect on bilirubin conjugation by affecting the glucosylation pathway. This is considered in detail in the Discussion section. Of the oestrogens tested, oestradiol 3-methyl ether, with only the 17-hydroxyl group available and the phenolic hydroxyl group blocked, was the most potent inhibitor, and this was true of both glucosylation and glucuronidation. Oestradiol was almost equally effective in inhibiting glucosylation, but was very much less effective as an inhibitor of glucuronidation. These observations suggest that, if oestradiol causes inhibition by acting as an alternative substrate for glucosylation, the 17-hydroxyl group is the preferred site for glucosylation, and it appears that blocking of the 3-hydroxyl group actually gives a better inhibitor (Table 4). Such a behaviour is in accord with the report of Collins *et al.* (1970), who found oestradiol 3-glucuronoside to be an excellent substrate for glucosyltransferase with K_m 7.14×10^{-8}M. The tested oestrogens inhibited *p*-nitrophenyl glucoside formation with decreasing effectiveness in the following order: oestradiol 3-methyl ether > oestradiol > oestriol > oestrone. *p*-Nitrophenyl glucuronide formation was inhibited with the following decreasing effectiveness: oestradiol 3-methyl ether > oestriol > oestradiol, but oestrone had very little effect.

We also tested the effect of the presence of UDP-glucuronic acid on glucosylation of *p*-nitrophenol and found it to be inhibitory (see Table 4). A glucosylation experiment was done at three different concentrations of UDP-glucose (4, 10 and 20mM) in the

presence and absence of 2mM-UDP-glucuronic acid. A double-reciprocal plot suggested a non-competitive inhibition for UDP-glucuronic acid. However, in view of the irregular kinetic behaviour noted in the experiments described above the significance of the last-mentioned observation cannot be assessed.

Discussion

The results reported here show that *p*-nitrophenol UDP-glucosyltransferase is primarily a microsomal enzyme. In liver whole homogenate as well as in microsomal preparations the ratio of glucosyltransferase activity to glucose 6-phosphatase activity fell within a narrow range (0.18–0.22), indicating a close association of the transferase with microsomal membranes. On the other hand, when post-mitochondrial fraction (post-5090*g*) was centrifuged at 25300*g* and then at 105000*g* the transferase activity sedimented partially at both these speeds together with particulate glycogen (which sediments principally at 25000*g*; Leloir & Goldemberg, 1960), and glucuronyltransferase (which sediments between 20000*g* and 105000*g*; Dutton, 1956; Halac & Frank, 1960).

It is noteworthy that the solubilized and partially purified rabbit liver microsomal oestrogen UDP-glucosyltransferase, reported by Collins *et al.* (1970), behaved differently from the *p*-nitrophenol glucosyltransferase studied here in that pH8.0 rather than pH7.0 appeared to be the optimum. However, Collins *et al.* (1970) pointed out that the shape of the pH curve was comparable with that found for steroid *N*-acetylglucosoaminyltransferase (Collins *et al.*, 1968). The latter curve appears to have an inflexion at about pH7.0 and then rises steeply to an optimum at pH8.0, and another inflexion occurs at about pH8.4 on the downward slope of the curve. Such behaviour is suggestive of a mixture of enzymes rather than a single enzyme. In view of the report by Williamson *et al.* (1971) that a UDP-glucose-independent steroid-glucosylating pathway is also present in rabbit liver microsomes, it seems likely that the pH profile obtained by Collins *et al.* (1970) was for a mixture of at least two enzymes, since they used tritiated steroid aglycone for their study and therefore could have been detecting steroid glucoside generated by UDP-glucoside-dependent and -independent pathways. By contrast, since we used UDP-[^{14}C]glucose, we were studying only the UDP-glucose-dependent pathway, which had a pH optimum at pH6.8–7.0. Further studies are needed to determine whether the UDP-glucose-dependent pathway exhibits an optimum at about pH7 for both *p*-nitrophenol and steroid substrates.

Our attempts at defining the kinetic behaviour of the *p*-nitrophenol glucosyltransferase lead us to the conclusion that the enzyme in our preparations does not obey simple Michaelis–Menten kinetics. It is noteworthy, and may be relevant to the behaviour of microsomal glucosyltransferase, that Winsnes (1969) in studies of *p*-nitrophenol glucuronyltransferase observed a similar deviation from Michaelis–Menten kinetics with fresh and unactivated preparations of microsomal glucuronyltransferase but found that the deviation was abolished in detergent-activated preparations. Our studies were carried out on unsolubilized fresh microsomal preparations. Moreover, we observed a sigmoid *p*-nitrophenol saturation curve for the glucosyltransferase, which raises the possibility of allosteric co-operative effects (Monod *et al.*, 1963; Gerhart & Pardee, 1964). In fact, both the deviation from Michaelis–Menten kinetics and the complex pH-dependence of glucosylation could be indicative of subunit structure of the enzyme and subunit interactions (Gerhart & Pardee, 1964). Further studies are needed to explore these possibilities.

To our knowledge this is the first report of inhibition of glucosyltransferase by steroids. In the light of the findings by Collins *et al.* (1970) of oestrogen glucoside conjugation, the inhibition of *p*-nitrophenol glucosylation by oestrogens is readily understandable. However, we also found that progestins, such as pregnane-3α,20β-diol, can act as inhibitors, which implies that progestins may also form glucosides. In any event, our observation that pregnane-3α,20β-diol was an inhibitor of glucosylation but not of glucuronidation is significant for the effect of the steroid on the conjugation of bilirubin, which is now known to undergo microsomal glucosylation (Wong, 1971) as well as glucuronidation (Dutton, 1966*a*). Our observations may explain some of the contradictory results reported in earlier studies of bilirubin conjugation. Attempts have been made to relate the inhibition of bilirubin glucuronyltransferase by hormonal steroids to incidence of some cases of neonatal jaundice (Lathe & Walker, 1958; Hsia *et al.*, 1960; Jones, 1964; Arias *et al.*, 1964; Gartner & Arias, 1964; Rosenfeld *et al.*, 1967; Krauer-Mayer *et al.*, 1968; Severi *et al.*, 1970; Adlard & Lathe, 1970*b*; Hargreaves & Piper, 1971; Wong & Wood, 1971). Good correlation has not always been observed, and especially controversial have been the findings about the effect of pregnane-3α,20β-diol, found as an unusual metabolite in lactating women, the presence of which appears to be associated with prolonged hyperbilirubinaemia in infants nursed by these mothers (Arias *et al.*, 1964; Rosenfeld *et al.*, 1967; Krauer-Mayer *et al.*, 1968; Severi *et al.*, 1970). There is a controversy about whether or not pregnane-3α,20β-diol inhibits bilirubin conjugation. Although the problem is related in part to species differences (Lathe & Walker, 1958; Adlard & Lathe, 1970*b*), differences in the assay conditions employed have also to be taken

into account, as well as the fact that bilirubin conjugation is not solely a glucuronidation process. Working with slices of rat or rabbit liver, Lathe & Walker (1958) and Bevan *et al.* (1965) found that pregnane-3α,20β-diol caused an inhibition of bilirubin conjugation; however, in assays where microsomal preparations of rat liver supplemented with UDP-glucuronic acid were used no inhibition by the steroid was observed (Adlard & Lathe, 1970*a*; Hargreaves & Piper, 1971). The last-mentioned workers also reported that pregnane-3α,20β-diol decreased the amount of conjugated bilirubin in the assay with rat liver slices, but they attributed this effect to an inhibition of the secretory process. On the basis of the results reported here, it seems likely that in assays with liver slices the effects on glucosylation come into play, and hence the results reported by others could be explained by an inhibition of the glucosylation pathway, which may or may not be involved in the secretory mechanism.

Heirwegh *et al.* (1971) and Wong (1971) found bilirubin UDP-glucosyltransferase in the microsomal fraction of rat liver. In many respects Wong's (1971) results on the glucosylation of bilirubin are comparable with ours obtained with *p*-nitrophenol as the model substrate. For instance, Wong (1971) found that a microsomal preparation was about four to five times more active in glucuronidation than in glucosylation; moreover, the points of the double-reciprocal plot reported by Wong (1971) appear to deviate from linearity in a manner similar to that observed in our studies, and also the K_m of 1.6mM for UDP-glucose is similar to the apparent K_m for UDP-glucose in the *p*-nitrophenol-glucosylation assay, when a concentration range comparable to that used by Wong (1971) is considered. In view of these analogies, we suggest that our results obtained on *p*-nitrophenol glucosylation may be generally applicable to the bilirubin-glucosylation pathway. It is noteworthy in this context that all the steroids tested by us were found to be more inhibitory to the glucosyltransferase than to glucuronyltransferase.

The present results, taken together with our earlier observation that *p*-nitrophenyl glucoside is only a very minor metabolite of *p*-nitrophenol in the urine (accounting for about 1% of a dose in a 5h urine collection; Gessner & Hamada, 1970), indicate that ordinarily glucosylation *in vivo* is a minor pathway of detoxication. At least two reasons for this are apparent: (*a*) microsomal glucosylation is much less active than glucuronidation (see Table 3), and (*b*) glucosyltransferase is subject to inhibition by UDP-glucuronic acid (see Table 4), endogenous concentrations of which probably are high most of the time. However, according to Wong's (1971) calculations the hepatic concentration of UDP-glucose is higher than that of UDP-glucuronic acid (1.4 and 0.4mM respectively; Wong, 1971). Moreover, Levy & Procknal (1968) found that glucuronide conjugation is a pathway of limited capacity, possibly owing to limited availability of UDP-glucuronic acid. The possibility therefore arises that, under conditions of saturation of the glucuronide-forming pathway or when concentrations of UDP-glucuronic acid are substantially lowered, glucosylation can gain in importance as an alternative pathway of detoxication.

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