Biochem. J. (1970) **118**, 625–634
Printed in Great Britain

Partial Purification and Kinetics of Oestriol 16α-Glucuronyltransferase from the Cytosol Fraction of Human Liver

BY GOVIND S. RAO, MARIE LUISE RAO AND HEINZ BREUER
Institut für Klinische Biochemie der Universität Bonn, 53 Bonn-Venusberg, Germany

(*Received* 11 *March* 1970)

An enzyme that conjugates the 16α-hydroxyl group of oestriol with glucuronic acid was found in the cytosol fraction of human liver. The enzymic activity could not be sedimented when the cytosol fraction was centrifuged at 158000$g_{av.}$ for 120min. The oestriol 16α-glucuronyltransferase was purified 100-fold by 0–30% saturation of the cytosol fraction with ammonium sulphate followed by filtration of the precipitate through Sephadex G-200. The activity was eluted at the void volume. The product of the reaction, oestriol 16α-monoglucuronide, was identified by paper chromatography and by crystallization of radioactive product to constant specific radioactivity. The optimum temperature was 37°C, and the activation energy was calculated to be 11.1kcal/mol. The apparent Michaelis–Menten constants for oestriol and UDP-glucuronic acid were 13.3 and 100 μM respectively. Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited, whereas Mg^{2+}, Mn^{2+} and Fe^{2+} stimulated the enzyme. Substrate-specificity studies indicated that the amount of oestradiol-17β, oestradiol-17α and oestrone conjugated was not more than about 5% of that found for oestriol. Oestriol 16α-monoglucuronide, a product of the reaction, did not inhibit the 16α-oestriol glucuronyltransferase; in contrast, UDP, another product of the reaction, inhibited the enzyme competitively with respect to UDP-glucuronic acid as the substrate, and non-competitively with respect to oestriol as the substrate. ATP and UDP-*N*-acetylglucosamine did not affect the oestriol 16α-glucuronyltransferase. 17-Epioestriol acted as a competitive inhibitor and 16-epioestriol as a non-competitive inhibitor of the glucuronidation of oestriol. 5α-Pregnane-3α,20α-diol also inhibited the enzyme non-competitively. It is most likely that the oestriol 16α-glucuronyltransferase described here is bound to the membranes of the endoplasmic reticulum.

The transfer of the glucuronic acid residue from UDP-glucuronic acid to various steroids is catalysed by glucuronyltransferases (EC 2.4.1.17). Conjugation with glucuronic acid is one of the major pathways that contributes to the 'detoxication' mechanisms in steroid metabolism. The transfer of glucuronic acid has been suggested by Slaunwhite, Lichtman & Sandberg (1964) to be a practical route for the biogenesis of oestrogen glucuronides.

The glucuronyltransferases appear to be associated with the microsomal fraction (Dutton, 1966), and solubilization of the microsomal enzymes is characterized by large losses of activity (Leventer, Buchanan, Ross & Tapley, 1965; Tomlinson & Yaffe, 1966). Isselbacher, Chrabas & Quinn (1962) were successful in solubilizing a microsomal glucuronyltransferase by treatment with snake venom; the enzyme, conjugating *p*-nitrophenol, was purified 30-fold. Lueders & Kuff (1967) observed spontaneous activation of the microsomal glucuronyltransferase, forming *p*-nitrophenol glucuronide, after addition of low concentrations of deoxycholate and Triton X-100.

Reports in the literature about glucuronyltransferases have dealt with the particulate enzyme and a wide variety of substrates (cf. Tomlinson & Yaffe, 1966). The occurrence of soluble oestriol glucuronyltransferases was first observed in the ground plasma (150000*g* supernatant) of human intestine (Dahm & Breuer, 1966). We have since found an enzyme in the high-speed supernatant fraction of the small intestine of the pig that shows high specificity towards oestradiol-17β (Rao & Breuer, 1969). The present paper describes the occurrence of glucuronyltransferase in the cytosol fraction of human liver; this enzyme conjugates predominantly oestriol in the 16α-position. Kinetic studies to characterize the enzyme are presented.

MATERIALS

Buffers. Glycine–NaOH, tris–HCl, tris–maleate and phosphate buffers were prepared as described by Gomori (1955). After the required dilutions had been made, the pH was checked with a Corning model 7 pH-meter. Chemicals for the preparation of buffers were obtained from E. Merck A.-G., Darmstadt, Germany.

Chemicals. [1,2-$^3H_2$]Aldosterone (11β,21-dihydroxy-3,20-dioxopregn-4-en-18-al; specific radioactivity 10Ci/mmol), [4-^{14}C]cortisol (11β,17α,20-trihydroxypregn-4-ene-3,20-dione; specific radioactivity 56.8mCi/mmol), [4-^{14}C]oestradiol-17β [oestra-1,3,5(10)-triene-3,17β-diol; specific radioactivity 51.4mCi/mmol], [4-^{14}C]oestriol [oestra-1,3,5(10)-triene-3,16α,17β-triol; specific radioactivity 51.4mCi/mmol], [6,7-$^3H_2$]oestrone [3-hydroxyoestra-1,3,5(10)-triene-17-one; specific radioactivity 36.8Ci/mmol] and 17α-hydroxy[4-^{14}C]progesterone (17α-hydroxypregn-4-ene-3,20-dione; specific radioactivity 35.9mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [6,7-$^3H_2$]Oestradiol-17α [oestra-1,3,5(10)-triene-3,17α-diol; specific radioactivity 4.0mCi/mmol] was kindly made available by Dr R. Knuppen, Bonn, Germany.

Oestradiol-17β, oestradiol-17α, oestriol, 16-epioestriol [oestra-1,3,5(10)-triene-3,16β,17β-triol], 17-epioestriol [oestra-1,3,5(10)-triene-3,16α,17α-triol] and oestrone were obtained from Schering A.-G., Berlin, Germany. 5α-Pregnane-3α,20α-diol was purchased from Fluka A.-G., Buchs, Switzerland. Oestriol monoglucuronides were generous gifts from Professor A. E. Kellie, London, U.K.

UDP-glucuronic acid disodium salt, UDP disodium salt, UDP-*N*-acetylglucosamine and ATP were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Bilirubin was a product of E. Merck A.-G., Darmstadt, Germany.

DEAE-cellulose and CM-cellulose were obtained from Serva, Heidelberg, Germany. Sephadex G-200, DEAE-Sephadex and CM-Sephadex were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxyapatite was obtained from Schuchardt, Munich, Germany.

EXPERIMENTAL

Preparation of the 158000g supernatant. The liver was obtained from the surgery unit during a kidney transplantation. The subject was a 25-year-old male who had taken an overdose of barbiturate and had been given dialysis treatment for 8h before the above operation was done.

The tissue was processed immediately. All operations were carried out at 0–4°C, unless otherwise stated. After washing off blood and cleaning the cut-up pieces with 0.25M-sucrose, the tissue pieces were homogenized with 7 parts (v/w) of 0.25M-sucrose in a Waring Blendor intermittently for 1min. The homogenate was centrifuged for 30min at 12000*g* in a refrigerated centrifuge. The supernatant was filtered through four layers of cotton gauze and centrifuged in a Beckman–Spinco model L-2 centrifuge for 1h at 158000$g_{av.}$. The high-speed supernatant thus obtained was transferred to fresh centrifuge tubes and recentrifuged for 1h at 158000*g*. No attempt was made to remove the thin yellow lipid layer floating on the top of the centrifuge tubes. The supernatant, obtained after two centrifugations at 158000$g_{av.}$, was designated the 'cytosol' fraction and stored in convenient portions at −20°C.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Protein concentrations were expressed in mg/ml from a standard curve obtained with dry bovine serum albumin.

Assay of glucuronyltransferase activity. The assay method was similar to that described by Rao & Breuer (1969). [4-^{14}C]Oestriol was used as substrate; it was purified in two different paper-chromatographic systems and diluted with recrystallized unlabelled oestriol to give 100000c.p.m., equivalent to 30nmol in 0.1ml of ethanol. To 0.1ml of this solution propylene glycol (2 drops) was added, and the ethanol was evaporated off under N_2 in a water bath at 40°C. To the residue was added 1.0ml of 0.05M-tris–HCl buffer, pH8.0, 0.1ml of a solution containing 1.0μmol of UDP-glucuronic acid, 0.1ml of a solution containing 20μmol of $MgCl_2$, and the enzyme preparation containing 100–200μg of protein. Incubations were carried out at 37°C for 1h. With each experiment, a control incubation was carried out with all the above ingredients except UDP-glucuronic acid.

Extraction, measurement of radioactivity and calculation. After incubation, the tubes were cooled in a bath of ice–water and the contents were extracted three times with ethyl acetate (5ml) saturated with water to remove quantitatively the unconjugated substrate. To the aqueous phase, containing the glucuronide, NaCl was added until a slight excess remained undissolved. The contents were extracted with butan-1-ol (2ml) saturated with water. A portion (0.5ml) of the butan-1-ol extract was pipetted into a counting vial; scintillation fluid (15ml) and methanol (1ml) were added. A Packard Tri-Carb model 3003 liquid-scintillation spectrometer was used for the determination of radioactivity. The scintillation fluid contained 5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of dry sulphur-free toluene. The settings for ^{14}C gave an efficiency of 70% when the above-mentioned mixture was used. The activity of the enzyme was calculated from the relationship:

$$\frac{(a-b)\times 30}{100000}$$

where a is c.p.m. in the butan-1-ol fraction of the test incubation and b is c.p.m. in the butan-1-ol fraction of the control incubation; 30 denotes nmol of substrate used and 100000 (c.p.m.) is the amount of radioactivity incubated.

Unit of enzyme activity. A unit of enzyme activity was defined as the amount necessary to catalyse the conjugation of 1nmol of oestriol/h under the standard assay conditions. Specific activity was defined as units/mg of protein.

The method described by Van Roy & Heirwegh (1968) was used to measure the glucuronidation of bilirubin. Pyrophosphatase was determined by the method of Ogawa, Sawada & Kawada (1966).

RESULTS

Purification of the enzyme

Ammonium sulphate precipitation. Preliminary experiments showed that glucuronyltransferase activity was present in the sediment obtained after 0–30% saturation of the cytosol fraction with ammonium sulphate. Ammonium sulphate was therefore added to the cytosol fraction to a saturation of 30%; the mixture was stirred for 15min and centrifuged at 12000*g* for 15min.

Gel filtration on Sephadex G-200. Sephadex G-200 was allowed to swell in water for 3–5 days and was washed first thoroughly with water and then with 2.5mM-tris–HCl buffer, pH 8.0. The gel was packed into a column (1.8cm × 90cm) in the same buffer, which was also used for elution. The precipitate obtained after 0–30% saturation of the cytosol fraction with ammonium sulphate was dissolved in 1.5–2.0ml of 2.5mM-tris–HCl buffer and applied to the Sephadex G-200 column. Fractions of volume 5ml were collected. Maximum specific activity of the glucuronyltransferase was found at the void volume. These fractions were combined and used immediately as the source of enzyme for kinetic studies. A typical purification of the oestriol 16α-glucuronyltransferase is shown in Table 1.

Attempts to purify the enzyme further were unsuccessful. It was observed that the fraction obtained after filtration through Sephadex G-200 could be adsorbed on a column of DEAE-cellulose or DEAE-Sephadex, but elution of the enzyme with a salt gradient could not be achieved. When a pH gradient was used, two peaks of protein, one at pH 6.4 and the other at pH 5.8, were obtained; however, both protein peaks were devoid of enzymic activity. Adsorption did not occur on CM-cellulose, CM-Sephadex or hydroxyapatite columns. The specific activity of the unadsorbed fraction did not show any increase.

Identification of oestriol 16α-monoglucuronide. The butan-1-ol extracts (glucuronide fraction) from several incubations of the enzyme with [4-^{14}C]-oestriol were collected and evaporated under nitrogen in a water bath at 40°C. The residue, containing the glucuronide and salts, was taken up in dry butan-1-ol, and the mixture was centrifuged for 10min at 3000*g* to sediment the salts. The butan-1-ol phase was removed and evaporated to dryness under nitrogen in a water bath at 40°C. The residue was chromatographed on paper (Schleicher und Schüll paper no. 2043b Mgl.) with authentic oestriol 16α-monoglucuronide as standard. The solvent system used for the first chromatography was 2-methylpropan-2-ol–ethylene dichloride–acetic acid–water (5:15:6:14, by vol.) (Smith & Breuer, 1963). After being dried, the chromatogram was scanned in a Packard model 7200 radiochromatogram scanner. The peak of radioactivity had the same mobility as that of authentic oestriol 16α-monoglucuronide, which was located by Folin–Ciocalteu reagent. Elution and rechromatography of the radioactive zone in the solvent system ethyl acetate–toluene–*n*-hexane–2-methylpropan-2-ol–acetic acid–water (12:15:8:5:12:28, by vol.) as described by Schneider & Lewbart (1959) showed excellent agreement between the peak of radioactivity and the authentic oestriol 16α-monoglucuronide. In further experiments the radioactive oestriol 16α-monoglucuronide fraction was hydrolysed with β-glucuronidase (Schering A.-G., Berlin); complete hydrolysis of the glucuronide was observed. Oestriol, which was obtained after hydrolysis, showed the same mobility as the authentic compound when subjected to paper chromatography in two different systems. In the presence of saccharolactone, a β-glucuronidase inhibitor, the hydrolysis of the glucuronide was inhibited by 40%.

Further evidence of the identity of the glucuronide with oestriol 16α-monoglucuronide was obtained by recrystallization to constant specific radioactivity. [4-^{14}C]Oestriol (1μCi) was incubated with UDP-glucuronic acid, magnesium chloride and the purified enzyme. The glucuronide formed was subjected to paper chromatography in the two different solvent systems described above. The radioactive oestriol 16α-monoglucuronide fraction obtained after the second chromatography was mixed with unlabelled oestriol 16α-monoglucuronide (3.03mg) and passed through a column of Sephadex

Table 1. *Purification of the oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver*

The enzyme preparations were incubated with [4-^{14}C]oestriol as described in the Experimental section. Further details of the purification of the enzyme are given in the text.

Fractions	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg of protein)	Purification
158000*g* supernatant	440	176	100	0.4	1
0–30%-satn.-$(NH_4)_2SO_4$ precipitate	31.2	87	50	2.8	7
Sephadex G-200 filtrate	6.1	54	31	38.7	97

Table 2. *Recrystallization to constant specific radioactivity of* [4-^{14}C]*oestriol* 16α*-monoglucuronide formed during incubation of* [4-^{14}C]*oestriol with the purified oestriol* 16α*-glucuronyltransferase from the cytosol fraction of human liver*

The solvent mixture used was methanol–water–dioxan. Values recorded are of specific radioactivity (c.p.m./mg).

Initial solution		First crystallization	Second crystallization	Third crystallization
7920	Crystals	7980	7350	7680
	Mother liquor	6700	7000	7090

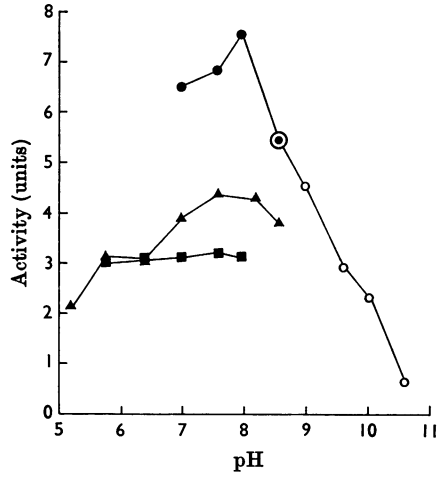

Fig. 1. Effect of pH on the formation of oestriol 16α-monoglucuronide by the purified oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver. The incubation mixture contained [4-^{14}C]oestriol (30nmol), UDP-glucuronic acid (1.0μmol) and buffer. The concentration of buffers was 0.05M. The following buffers were used: ■, phosphate buffer (pH5.7–8.0); ▲, tris–maleate buffer (pH5.2–8.6); ●, tris–HCl buffer (pH7.2–8.6); ○, glycine–NaOH buffer (pH8.6–10.7). The formation of [4-^{14}C]oestriol 16α-monoglucuronide was measured as described in the Experimental section.

G-25. The glucuronide was eluted with water. Fractions containing the glucuronide were combined and the water was evaporated off under nitrogen in a water bath at 45°C. The residue was crystallized to constant specific radioactivity. The results are presented in Table 2.

Properties of the purified enzyme

Stability. The purified oestriol 16α-glucuronyltransferase was stable for more than 2 weeks at −20°C, whereas it lost 75% of its initial activity after 3 days when kept at +4°C. Attempts to stabilize the enzyme with glycerol or albumin were not successful. Incorporation of cysteine or mercaptoethanol during the purification steps yielded lower specific activities and rendered the enzyme more unstable.

Effect of pH *on enzyme activity.* Maximum activity was found at pH 8.0 with tris–HCl buffer (Fig. 1). In tris–maleate and phosphate buffers no sharp pH optimum was observed. Enzyme activity in these buffers was also low.

Effects of protein concentration and time. The formation of oestriol 16α-monoglucuronide was linear with up to 200μg of protein and for 80min. Further increase in protein concentration or time did not result in a proportional increase in enzymic activity.

Effect of incubation temperature on the formation of oestriol 16α*-monoglucuronide.* The enzyme showed maximal activity at 37°C. The activation energy, calculated by the formula for two temperatures, 27 and 37°C (White, Handler & Smith, 1964), was 11.1kcal/mol.

K_m for oestriol and UDP-glucuronic acid. Values for K_m were obtained by using Lineweaver & Burk (1934) plots. The K_m values for oestriol at saturating concentration of UDP-glucuronic acid and for UDP-glucuronic acid at saturating concentration of oestriol were calculated to be 13.3μM and 100μM respectively (Figs. 2*a* and 2*b*).

Substrate specificity. Appropriate amounts of different radioactive steroids in ethanol were pipetted into different tubes and prepared for incubation as described under 'Assay of glucuronyltransferase activity' (Table 3). A control incubation without UDP-glucuronic acid was carried out for each steroid used as substrate.

Effects of metal ions. Cu^{2+}, Zn^{2+} and Hg^{2+} inhibited the formation of oestriol 16α-monoglucuronide (Table 4). Mg^{2+}, Mn^{2+} and Fe^{2+} increased the rate of glucuronide formation. Although Mn^{2+} and Fe^{2+} appeared to stimulate the enzymic glucuronidation, they were found to be unsuitable for routine use. Mg^{2+} was used as a routine, since the stimulation was almost the same as that obtained with Mn^{2+} and Fe^{2+}.

Effect of products of the reaction on the oestriol 16α*-glucuronyltransferase.* UDP is one of the products of enzymic glucuronidation (Dutton,

1966). Its effect on the enzyme was studied (*a*) by varying the concentration of oestriol at saturating concentration of UDP-glucuronic acid and (*b*) by varying the concentration of UDP-glucuronic acid at saturating concentration of oestriol. The results obtained are presented in the form of Lineweaver–Burk plots in Figs. 3(*a*) and 3(*b*). With respect to oestriol (Fig. 3*a*) UDP caused a non-competitive inhibition with K_i 12.9 μM. With respect to UDP-glucuronic acid the inhibition was of the competitive type with K_i 4.42 μM (Fig. 3*b*).

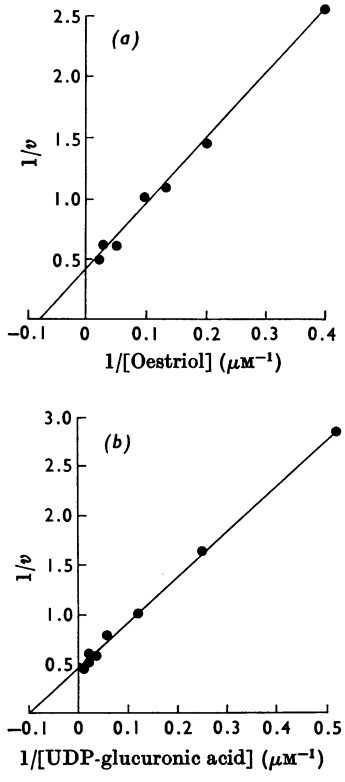

Fig. 2. Double-reciprocal plot of initial velocity against various concentrations of (*a*) oestriol and (*b*) UDP-glucuronic acid. The formation of [4-^{14}C]oestriol 16α-monoglucuronide was measured as described in the Experimental section. The activity, *v*, is expressed as μM-glucuronide.

The other product of enzymic glucuronidation is oestriol 16α-monoglucuronide. To study its effect on the enzyme, 2.5 μM-oestriol 16α-monoglucuronide was incubated with various concentrations of [4-^{14}C]oestriol at saturating UDP-glucuronic acid concentration and with various amounts of UDP-glucuronic acid at saturating oestriol concentration. The results derived from Lineweaver–Burk plots indicate that, at the concentration of oestriol 16α-monoglucuronide used, the initial and maximal velocities of the reaction were not altered to any significant extent.

Effect of ATP, 3′:5′-cyclic-AMP and UDP-N-acetylglucosamine on the oestriol 16α-glucuronyltransferase. 3′:5′-Cyclic-AMP had no effect on the glucuronidation of oestriol at final concentrations of 50, 250 and 500 μM. Similarly, ATP and UDP-*N*-acetylglucosamine (50, 250 and 500 μM) did not enhance the activity of the oestriol 16α-glucuronyltransferase. UDP-*N*-acetylglucosamine, when used without UDP-glucuronic acid, did not lead to any conjugation.

ATP and UDP-*N*-acetylglucosamine are known to stimulate the formation of *p*-nitrophenol glucuronide by rat liver microsomes; this stimulation has been attributed to the prevention of the breakdown of UDP-glucuronic acid by the microsomal pyrophosphatases (Pogell & Leloir, 1961). On using the method of Ogawa *et al.* (1966), no pyrophosphatase activity could be detected in the partially purified enzyme.

Table 3. *Substrate specificity of the purified oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver*

Appropriate amounts of the indicated steroids in ethanol were pipetted into tubes; propylene glycol (2 drops) was added and the ethanol was evaporated off under a stream of N_2 in a water bath. To each tube 1.0ml of 0.05M-tris–HCl buffer, pH8.0, UDP-glucuronic acid (1.0μmol) and the enzyme were added and the mixture was incubated for 1h at 37°C. Formation of the radioactive glucuronides was measured as described in the Experimental section. Relative conjugations were obtained by taking the conjugation of [4-^{14}C]oestriol as 100%.

Substrate	Radioactivity incubated (c.p.m.)	Amount of substrate conjugated (%)	Relative conjugation (%)
[1,2-$^3H_2$]Aldosterone	219000	0	0
[4-^{14}C]Cortisol	90000	0.15	1
17α-Hydroxy[4-^{14}C]progesterone	175000	0	0
[4-^{14}C]Oestradiol-17β	78000	0.90	6
[6,7-$^3H_2$]Oestradiol-17α	1900000	0.4	2.6
[4-^{14}C]Oestriol	117000	15.0	100
[6,7-$^3H_2$]Oestrone	510000	0.35	2.3

Table 4. *Effect of metal ions on the purified oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver*

The rate of formation of oestriol 16α-monoglucuronide was measured in the presence of the different metal ions as shown below. The incubation mixture contained [4-^{14}C]oestriol (30nmol), UDP-glucuronic acid (1.0μmol) and 1.0ml of 0.05M-tris–HCl buffer, pH8.0. The formation of oestriol 16α-monoglucuronide was measured as described in the Experimental section. The rate of glucuronide formation is expressed as percentage of the control rate.

Metal ion added	Concentration (mM)	Rate of glucuronide formation (%)
None (control)	—	100
Mg^{2+}	0.5	141
	2.5	151
Mn^{2+}	0.5	166
	2.5	161
Cu^{2+}	0.5	22
	2.5	3
Ca^{2+}	0.5	126
	2.5	146
Zn^{2+}	0.5	0
	2.5	0
Hg^{2+}	0.5	0
	2.5	0
Fe^{2+}	0.5	151
	2.5	146

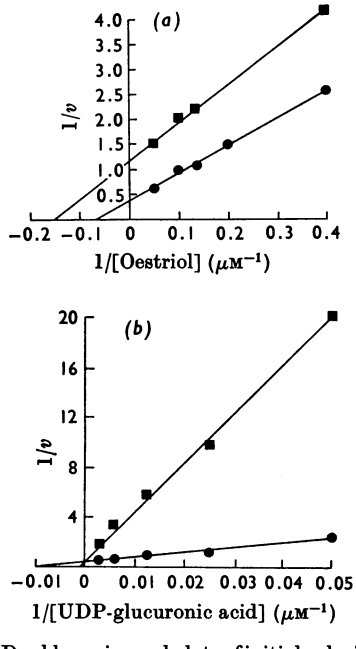

Fig. 3. (*a*) Double-reciprocal plots of initial velocity against variable oestriol concentration. ●, Without UDP; ■, in the presence of 15μM-UDP. (*b*) Double-reciprocal plots of initial velocity against various UDP-glucuronic acid concentrations. ●, Without UDP; ■, in the presence of 15μM-UDP. The activity, *v*, is expressed as μM-glucuronide.

Effect of some steroids on the oestriol 16α-glucuronyltransferase. Oestradiol-17β, oestradiol-17α and oestrone are also conjugated by the oestriol 16α-glucuronyltransferase, but only to a small extent (Table 3). To test whether these steroids are conjugated by the same enzyme, the following steroids were tested for their effect on the formation of oestriol 16α-monoglucuronide: oestradiol-17β, oestradiol-17α, oestrone, 17-epioestriol, 16-epioestriol and 5α-pregnane-3α,20α-diol. Figs. 4(*a*)–4(*f*) show the results in the form of double-reciprocal plots of the velocity against concentration of oestriol at saturating concentration of UDP-glucuronic acid in the presence of different amounts of the above steroids. The K_i values, calculated from the data obtained from the double-reciprocal plots, as well as the type of inhibition, are summarized in Table 5.

DISCUSSION

The experiments reported here demonstrate the occurrence of an enzyme system in the cytosol fraction of human liver that is capable of conjugating the 16α-hydroxyl group of oestriol with glucuronic acid. This enzyme, which may be called an oestriol 16α-glucuronyltransferase, can be purified about 100-fold by 30% saturation of the cytosol fraction with ammonium sulphate and filtration of the ammonium sulphate precipitate through Sephadex G-200. At this stage of purifica-

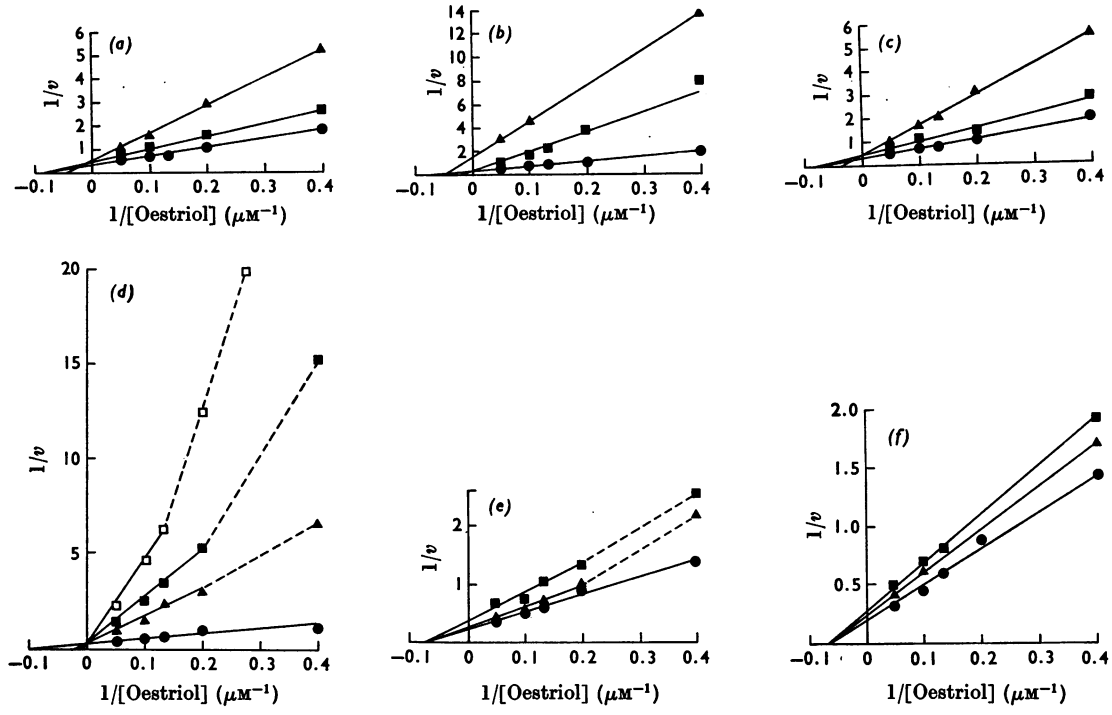

Fig. 4. Double-reciprocal plots of initial velocity against variable oestriol concentrations. (*a*) ●, No addition; ■, in the presence of 2.5μM-oestradiol-17β; ▲, in the presence of 10μM-oestradiol-17β. (*b*) ●, No addition; ■, in the presence of 2.5μM-oestradiol-17α; ▲, in the presence of 10μM-oestradiol-17α. (*c*) ●, No addition; ■, in the presence of 2.5μM-oestrone; ▲, in the presence of 10μM-oestrone. (*d*) ●, No addition; ▲, in the presence of 2.5μM-17-epioestriol; ■, in the presence of 5μM-17-epioestriol; □, in the presence of 10μM-17-epioestriol. (*e*) ●, No addition; ▲, in the presence of 2.5μM-16-epioestriol; ■, in the presence of 10μM-16-epioestriol. (*f*) ●, No addition; ▲, in the presence of 2.5μM-5α-pregnane-3α,20α-diol; ■, in the presence of 5.0μM-5α-pregnane-3α,20α-diol. The activity, *v*, is expressed as μM-glucuronide.

Table 5. *Effect of some steroids on the purified oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver*

Incubation mixtures containing [4-^{14}C]oestriol in various concentrations, UDP-glucuronic acid (1.0μmol), 1.0ml of 0.05M-tris–HCl buffer, pH8.0, and steroids at the concentrations indicated were incubated at 37°C. After 1h the incubations were stopped by cooling in ice–water and the rate of glucuronidation was measured as described in the Experimental section. The K_i values were calculated from data obtained from Figs. 4(*a*)–4(*f*).

Steroid	Concentration (μM)	Type of inhibition	K_i (μM)
Oestrone	2.5	Non-competitive	5.0
	10.0	Non-competitive (mixed)	
Oestradiol-17β	2.5	Non-competitive	5.0
	10.0	Non-competitive (mixed)	
Oestradiol-17α	2.5	Competitive	0.93
	10.0	Non-competitive (mixed)	
5α-Pregnane-3α,20α-diol	2.5		
	10.0	Non-competitive	13.9
17-Epioestriol	2.5		
	10.0	Competitive	0.78
16-Epioestriol	2.5		
	10.0	Non-competitive	13.9

tion the enzyme shows a high degree of instability and has to be used immediately for further studies. Addition of glycerol or albumin has no stabilizing effect. Mercaptoethanol and cysteine decrease the stability of the oestriol 16α-glucuronyltransferase. These effects could indicate that the enzyme does not require thiol groups for activity. The formation of oestriol 16α-monoglucuronide is stimulated by the addition of Mn^{2+}, Fe^{2+} and Mg^{2+}; however, these metal ions cannot stabilize the enzyme. Zn^{2+} and Hg^{2+} totally inhibit the oestriol 16α-glucuronyltransferase, and Cu^{2+} at a concentration of 2.5μM produces an almost total inhibition.

So far, very little has been reported about the properties of oestriol 16α-glucuronyltransferase (cf. Dahm & Breuer, 1966). The results of the kinetic studies, obtained with the 100-fold purified enzyme from the cytosol fraction of the human liver, reveal some interesting properties. The oestriol 16α-glucuronyltransferase shows a pH optimum of 8.0 in tris–HCl buffer. Dutton & Storey (1954), who used mouse liver homogenate, found a maximum at pH 7.6–7.9, in veronal–acetate buffer, for the glucuronidation of *o*-aminophenol. This finding is in good agreement with our value of pH 8.0. The activity of the oestriol 16α-glucuronyltransferase in phosphate buffer was low and did not show a well-defined maximum. Slaunwhite *et al.* (1964), who studied the 16α-glucuronidation of oestriol in human liver homogenate, obtained a pH optimum of 7.0 in phosphate buffer. This apparent difference is most probably due to the fact that in the present investigation a purified enzyme preparation was used.

The formation of oestriol 16α-monoglucuronide showed a linear relationship with time up to 80 min, and a zero-order reaction was observed. The temperature optimum was found to be 37°C. The activation energy was calculated to be 11.1 kcal/mol. The oestriol 16α-glucuronyltransferase from the cytosol fraction of the human liver shows normal Michaelis–Menten kinetics with respect to both substrates, oestriol and UDP-glucuronic acid.

Substrate-specificity studies with the oestriol 16α-glucuronyltransferase revealed that oestradiol-17β, oestradiol-17α and oestrone are also conjugated to a small extent. Aldosterone and 17α-hydroxyprogesterone were not conjugated, whereas cortisol was a poor substrate for the enzyme. In order to find out whether (1) the enzyme conjugates only a certain group of substrates, or (2) is specific for one substrate, or (3) specifically reacts with a functional group in the steroid molecule, several experiments were carried out. These experiments consisted of incubating two or three fixed concentrations of the compound of interest with various concentrations of oestriol in the presence of a saturating UDP-glucuronic acid concentration. The results were put in the form of double-reciprocal plots and the results analysed (Fig. 4 and Table 5).

17-Epioestriol (at 2.5, 5.0 and 10μM) and oestradiol-17α (at 2.5μM) produce competitive inhibition. This indicates that these steroids may be competing with oestriol for the same binding site on the enzyme. However, oestradiol-17α, when present at a concentration of 10μM, produces non-competitive inhibition. This change in the type of inhibition, at a concentration four times that at which a competitive inhibition is observed, is difficult to explain. On the basis of these results it is reasonable to assume that hydroxyl groups in the α-position in ring D of the steroid molecule may act as orienting structures for the attachment to the enzyme. Phenolic steroids tested, such as oestrone, oestradiol-17β and 16-epioestriol, are effective only as non-competitive inhibitors, suggesting that these steroids, which possess the basic oestrogen structure but are devoid of an α-hydroxyl group in ring D, bind to different sites on the enzyme. 17α-Hydroxyprogesterone and cortisol are not conjugated by the oestriol 16α-glucuronyltransferase. This could be attributed to the side chain at C-17, which possibly hinders the attachment of the enzyme to the steroid molecule.

From these results it may be concluded that the oestriol 16α-glucuronyltransferase is a group-specific enzyme attacking predominantly the 16α-hydroxyl group of the oestrogen molecule. Whether the same enzyme also catalyses the glucuronidation of 17-epioestriol cannot be ascertained from the experiments described above.

Previous investigations of the effect of UDP, which is one of the products of the enzymic glucuronidation, on the oestradiol-17β glucuronyltransferase from the pig intestine (Rao & Breuer, 1969) showed that UDP exhibited certain inhibitory properties. Therefore it seemed reasonable to study also the effect of UDP on the oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver. As shown in Fig. 3, UDP may act as a competitive as well as a non-competitive inhibitor, depending on the substrate that is varied. When oestriol is used at different concentrations, UDP (15μM) binds to the enzyme and produces a non-competitive inhibition; however, when the concentration of UDP-glucuronic acid is varied UDP acts as a competitive inhibitor. The dual type of inhibition by UDP with respect to the two substrates could be explained as follows. The competitive inhibition of the enzymic glucuronidation by UDP at saturating oestriol concentration may be due to the structural similarity of the two nucleotides, UDP and UDP-glucuronic acid. As the concentration of UDP-glucuronic acid is increased and reaches saturation, the inhibition is reversed and maximal velocity is attained. However, at saturat-

ing UDP-glucuronic acid concentration and at low initial oestriol concentration UDP introduces a non-competitive inhibition; since a further increase of oestriol has no influence, this inhibition may possibly be explained by an irreversible change in the conformation of the enzyme protein. Oestriol 16α-monoglucuronide, which is the other reaction product, has no inhibitory effect on its formation, irrespective of whether the concentrations of oestriol or UDP-glucuronic acid are altered.

It has been reported that ATP competitively inhibits the soluble oestradiol-17β glucuronyltransferase from pig intestine with respect to UDP-glucuronic acid as the substrate (Rao & Breuer, 1969). Pogell & Leloir (1961) observed that ATP, UDP-*N*-acetylglucosamine, or both, stimulate the glucuronidation of *p*-nitrophenol by rat liver microsomes, and they suggested that the two nucleotides prevent the breakdown of UDP-glucuronic acid by the microsomal pyrophosphatase. Slaunwhite *et al.* (1964) found an increase in the formation of oestriol glucuronide when UDP-*N*-acetylglucosamine was included in the incubation of homogenates of human liver with UDP-glucuronic acid and oestriol.

The influence of ATP and UDP-*N*-acetylglucosamine on the oestriol 16α-glucuronyltransferase from the cytosol fraction of human liver was studied. With the three different concentrations used (50, 250 and 500μM), both ATP and UDP-*N*-acetylglucosamine neither stimulated nor inhibited the glucuronidation of oestriol. UDP-*N*-acetylglucosamine alone did not form a conjugate, which indicates the absence of a UDP-*N*-acetylglucosaminyltransferase in the purified cytosol fraction of the human liver. The fact that UDP-*N*-acetylglucosamine did not stimulate the glucuronidation may indicate that UDP-glucuronic acid pyrophosphatase does not occur in the purified cytosol fraction of the human liver. The method used to detect pyrophosphatase (Ogawa *et al.* 1966) did not reveal the presence of the enzyme.

These results indicate that ATP, UDP-*N*-acetylglucosamine, or both, may react differently with different glucuronyltransferases and that glucuronyltransferases may be quite different, structurally as well as in their properties, from one another.

Although the activity of the oestriol 16α-glucuronyltransferase is comparatively low in the cytosol fraction, it can be purified. Since it is known that glucuronyltransferases occur in the microsomal fraction (Dutton, 1954; Isselbacher, 1956) and attempts to solubilize the microsomal enzymes often lead to inactivation, we considered the following as possible origins of the oestriol 16α-glucuronyltransferase in the cytosol fraction. (1) The oestriol 16α-glucuronyltransferase exists as such in the cytoplasm of the liver cell. (2) The enzyme that is found in the cytosol fraction is only loosely bound to the microsomes and liberated during homogenization of the tissue. (3) The procedure used to homogenize the tissue disrupts the subcellular organelles harbouring the enzyme and therefore the enzyme is found in the cytosol fraction. (4) Homogenization procedures break up the endoplasmic reticulum and release fragments to which the enzyme is bound; these extremely small fragments may not be sedimented by conventional centrifugation methods, and the enzyme activity remains in the cytosol fraction. This possibility appears to us to be the more likely one. Recent investigations (G. S. Rao, M. L. Rao & H. Breuer, unpublished work) with caesium chloride–sucrose-density-gradient centrifugations have revealed most of the activity to be present in two zones normally occupied by smooth-surfaced microsomes (Dallner, Siekevitz & Palade, 1966). Lueders & Kuff (1967) have also found that the enzyme catalysing the glucuronidation of *p*-nitrophenol remained completely bound to the microsomal membranes during both spontaneous and detergent-induced activation. From the four possibilities mentioned it is evident that further experiments are necessary to trace the origin of the oestriol 16α-glucuronyltransferase in the cytosol fraction.

As already pointed out, attempts to solubilize microsomal glucuronyltransferase have not been very successful. In spite of this technical difficulty, it would be desirable to investigate whether similarities exist between the microsomal enzymes that catalyse the conjugation of a number of steroids with glucuronic acid, and the cytoplasmic enzyme. Moreover, if the cytoplasmic enzyme is in fact of microsomal origin, then the question arises of why only the highly specific oestriol 16α-glucuronyltransferase, but not other glucuronyltransferases, occurs in the cytosol fraction.

This work was supported by the Deutsche Forschungsgemeinschaft and by the Bundesministerium für Wissenschaftliche Forschung. We thank Professor A. E. Kellie for his generous gift of steroid glucuronides. We are grateful to Professor S. Ichii for helpful discussion and Miss D. Urbach for technical assistance.

REFERENCES

Dahm, K. & Breuer, H. (1966). *Biochim. biophys. Acta*, **128**, 306.

Dallner, G., Siekevitz, P. & Palade, G. E. (1966). *J. Cell Biol.* **30**, 73.

Dutton, G. J. (1954). *Biochem. J.* **64**, 693.

Dutton, G. J. (1966). In *Glucuronic Acid, Free and Combined*, chapter 3, p. 217. Ed. by Dutton, G. J. New York: Academic Press Inc.

Dutton, G. J. & Storey, I. D. E. (1954). *Biochem. J.* **57**, 275.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Isselbacher, K. J. (1956). *Recent Prog. Hormone Res.* **12**, 134.

Isselbacher, K. J., Chrabas, M. F. & Quinn, R. C. (1962). *J. biol. Chem.* **327**, 3033.

Leventer, L. L., Buchanan, J. L., Ross, J. E. & Tapley, D. F. (1965). *Biochim. biophys. Acta*, **110**, 428.

Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Lueders, K. K. & Kuff, E. L. (1967). *Archs Biochem. Biophys.* **120**, 198.

Ogawa, H., Sawada, M. & Kawada, M. (1966). *J. Biochem., Tokyo*, **59**, 126.

Pogell, B. M. & Leloir, L. F. (1961). *J. biol. Chem.* **236**, 293.

Rao, G. S. & Breuer, H. (1969). *J. biol. Chem.* **244**, 5521.

Schneider, J. J. & Lewbart, M. L. (1959). *Recent Prog. Hormone Res.* **15**, 201.

Slaunwhite, W. R., jun., Lichtman, M. A. & Sandberg, A. A. (1964). *J. clin. Endocr.* **24**, 638.

Smith, E. R. & Breuer, H. (1963). *Biochem. J.* **88**, 168.

Tomlinson, G. A. & Yaffe, S. J. (1966). *Biochem. J.* **99**, 507.

Van Roy, F. P. & Heirwegh, K. P. M. (1968). *Biochem. J.* **107**, 507.

White, A., Handler, P. & Smith, E. L. (1964). In *Principles of Biochemistry*, 3rd ed., pp. 228, 229. New York: McGraw–Hill Book Co.