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Steroid Glucuronyltransferases of Rat Liver

PROPERTIES OF OESTRONE AND TESTOSTERONE GLUCURONYLTRANSFERASES AND THE EFFECT OF OVARIECTOMY, CASTRATION AND ADMINISTRATION OF STEROIDS ON THE ENZYMES

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1. Microsomal preparations from rat liver, kidney and intestine were tested for UDP-glucuronyltransferase activity by using oestrone, oestradiol-17β, oestriol, testosterone, cortisol, cortisone, corticosterone, aldosterone, tetrahydrocortisol and tetrahydrocortisone as substrates. The microsomal preparation from the liver glucuronidated oestrone, oestradiol-17β and testosterone. 2. The specific activity of the enzyme was significantly higher in livers from female rats than in those from male rats. 3. Testosterone was actively glucuronidated by both sexes. Cortisol, cortisone, corticosterone, aldosterone, tetrahydrocortisol and tetrahydrocortisone were not glucuronidated by any of the three tissues. 4. The non-ionic detergent Lubrol WX activates liver microsomal UDP-glucuronyltransferase 2–3-fold with oestrone and testosterone as substrates. 5. Oestrone glucuronyltransferase was inhibited by oestradiol-17β, predominantly competitively and by testosterone non-competitively. Bilirubin was a non-competitive inhibitor of oestrone glucuronidation. *p*-Nitrophenol had no effect. 6. Oestrone glucuronyltransferase could not be stimulated by either acute or prolonged treatment of animals with phenobarbital, whereas a single dose of 3-methylcholanthrene led to a moderate stimulation. 7. Ovariectomy leads to a 56% decrease in oestrone glucuronyltransferase activity; administration of oestradiol-17β induces the enzyme to normal activity after 12 days, and after 15 days the activity is twice the control value. Actinomycin D and cycloheximide block the oestradiol-17β-induced increase in enzyme activity. 8. Castration has no effect on the activity of testosterone glucuronyltransferase, nor does administration of testosterone influence enzyme activity. The results provide strong evidence for the existence of multiple steroid glucuronyltransferases in the liver of the rat.

Oestrogen glucuronyltransferase (UDP-glucuronate–17β-oestradiol 3-glucuronyltransferase, EC 2.4.1.59) catalyses the transfer of glucuronic acid from UDP-glucuronate to oestrone. In studies *in vitro*, foreign compounds such as *p*-nitrophenol, *o*-aminophenol and phenolphthalein have often been used as substrates for glucuronyltransferases to measure enzyme activity (Dutton, 1971; Winsnes, 1973). Endogenous substrates include bilirubin (Mulder, 1972) and steroid hormones such as oestrone, oestradiol-17β, oestriol, testosterone (Rao & Breuer, 1969; Rao *et al.*, 1970*a*,*b*, 1972, 1974; Götze *et al.*, 1971) and tetrahydrocorticosterone (Miller *et al.*, 1973). With so many different acceptors, it may be asked what degree of specificity UDP-glucuronyltransferase possesses. Our own previous work with steroid substrates, and other evidence (see Dutton, 1971; Jacobson *et al.*, 1975), suggests multiplicity of the enzyme.

As UDP-glucuronyltransferases are inducible (see Conney, 1967), the effects of administration of phenobarbital and 3-methylcholanthrene, two commonly used enzyme-inducing agents, on steroid glucuronyltransferases were investigated. Moreover, the possibility exists that therapy with steroid hormones or the use of both oestrogens and progestrogens for preventing pregnancy could result in increased transferase activity toward these hormones. Hence induction of enzyme activity by its substrate should give some information about the factors controlling the concentration of endogenous steroid hormones and their effect on enzyme activity. Therefore we decided to study the effect of ovariectomy and castration, followed by adminis-

tration of oestradiol-17β and testosterone, on UDP-glucuronyltransferases by using endogenous substrates such as oestrone and testosterone. In addition the information obtained from these studies would also provide evidence to support the concept of multiplicity of steroid glucuronyltransferases.

Experimental

Materials

Radioactive steroids used in this investigation were purchased from The Radiochemical Centre, Amersham, Bucks., U.K., or New England Nuclear Chemicals G.m.b.H., Dreieichenhain, Germany. Non-labelled steroids were from Merck, Darmstadt, Germany, and UDP-glucuronic acid was from Boehringer, Mannheim, Germany. 3-Methylcholanthrene, phenobarbital sodium, actinomycin D and cycloheximide were purchased from either Merck, Darmstadt or Serva, Heidelberg, Germany. Lubrol WX was generously given by ICI, Wilmslow, Cheshire, U.K. Other chemicals and solvents were obtained through local dealers.

Animals

Wistar rats weighing between 150 to 200g were kept in a room at 23°C with equal periods of daylight and darkness. The animals had free access to food and water; not more than three animals were kept in a single cage. All animals were starved for 24h before being killed.

Preparation of microsomal fractions from the small intestine, liver and kidney of the rat

The animals were killed by cervical dislocation; the small intestine, liver and both kidneys were quickly removed and transferred to ice-cold 0.25M-sucrose in 0.1M-Tris/HCl buffer, pH7.4. All procedures after this operation were carried out at 2–4°C. The small intestine was cleaned by forcing the buffered sucrose through the lumen with a suitable syringe. Extraneous tissue was trimmed from the liver and kidneys, which were then blotted and weighed. The tissues were cut into small pieces with scissors and homogenized separately in the buffered sucrose with an Ultra-Turrax instrument (Janke und Kunkel, Staufen, Germany) at half-maximal speed, intermittently, for a total of 1–2min. The homogenates were centrifuged in a cooled centrifuge (4°C) at 600*g* for 10min (8×90ml rotor, 2800rev./min). The supernatants were decanted into fresh tubes and centrifuged at 25000*g* for 10min. The microsomal fractions were sedimented by centrifuging the 25000*g* supernatants in a Beckman L2-65B ultracentrifuge at 169000*g* for 30min (8×38.5ml, rotor 60 Ti, 49000rev./min). The supernatants were discarded and the sediments were resuspended in the buffered sucrose by gentle homogenization in an all-glass homogenizer and resedimented at 169000*g* for 30min. The sediment was resuspended as described in the previous step in buffered sucrose and kept at 2–4°C for immediate use. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Behringwerke, Marburg, Germany) as standard.

Screening of the microsomal fractions from the small intestine, liver and kidney for transferase activity with oestrogens and androgen as substrates

Incubation with oestrogens and testosterone. The enzyme activity of the microsomal fractions was tested by incubating different radioactive steroids and UDP-glucuronic acid. Ethanolic solutions of the steroids were pipetted into tubes, one drop of propylene glycol was added and the ethanol evaporated with a gentle stream of N_2; 1ml of 0.1M-Tris/HCl buffer, pH7.4, 4mM-$MgCl_2$ and 2mM-UDP-glucuronic acid were added to each tube. The incubation was started by pipetting the microsomal suspension equivalent to 100–200μg of protein and shaking the tubes in a water bath at 37°C for 60min under air.

Extraction and chromatography of the glucuronides. After incubation the tubes were cooled in ice/water; solid NaCl was added, ensuring that a slight amount remained undissolved. The contents were extracted twice with 2ml of water-saturated butan-1-ol. The extracts were combined and evaporated under vacuum to dryness with a Rotavapor apparatus (Buchler Instruments, Flawil, Switzerland) in a water bath at 40°C. The residue was shaken with 3ml of dry butan-1-ol for 30–60min and briefly centrifuged (approx. 1000*g* for 2min) to sediment insoluble material. The butan-1-ol was carefully removed by disposable pipettes and transferred to tubes and evaporated to dryness. The residue was dissolved in a few drops of an elution mixture of butan-1-ol/methanol/water (1:2:1, by vol.), applied to paper and chromatographed in the solvent system ethyl acetate/toluene/*n*-hexane/2-methylpropan-2-ol/acetic acid/water (12:15:8:5:12:28, by vol.; Schneider & Lewbart, 1959) after equilibration overnight. The chromatogram was taken out when the solvent front had reached the end of the paper, dried for 30min in an oven at 60°C and scanned in a Packard paper-chromatogram scanner. This first chromatography step separated the glucuronides, which remain on the origin, from the unchanged steroids which move almost with the solvent front. The peaks of radioactivity on the origin were eluted from the paper with the elution mixture; the eluates were evaporated under vacuum to dryness and the residues rechromatographed in the same solvent system for different length of time to give adequate separation. The appropriate authentic steroid glucuronides were chromatographed as standards for

identification of the radioactive glucuronides. Final confirmation of the identity of the glucuronides of oestrone, oestradiol-17β, oestriol and testosterone was achieved by g.l.c. and mass spectrometry of the purified products with authentic standards.

Incubation of glucocorticoids. The transferase activity of the microsomal fractions from the three tissues (liver, kidney and intestine) with cortisol, cortisone, corticosterone and aldosterone as substrates was tested by incubating 5–10μCi of 1,2-^{3}H-labelled steroid, 10nmol of the non-labelled steroid and 1.5mM-UDP-glucuronic acid in 0.1M-Tris/HCl buffer, pH7.4, at 37°C for 60min. The incubations were worked up as described for oestrogens and testosterone. The solvent system for paper chromatography of the products was butan-1-ol/ethyl acetate/water (4:1:5, by vol.).

Operation on animals

Bilateral ovariectomy or castration was performed by the same person on one day; sexually mature animals were used. The operation was carried out under light diethyl ether anaesthesia under adequately sterile conditions. The animals were used for experiments 17–19 days after operation.

Assay of UDP-glucuronyltransferase activities

A rapid and economic assay to measure oestrone and testosterone glucuronyltransferase activities of microsomal preparations of the liver has been reported (Rao *et al.*, 1976). Briefly, the method consists of incubation of the microsomal fraction with the radioactive steroid (100000c.p.m.), diluted with the appropriate amount of non-labelled steroid (30μM-oestrone and 600μM-testosterone), 1 or 2mM-UDP-glucuronic acid and 10mM-$MgCl_2$ in 0.1M-Tris/HCl buffer, pH8.8, for 30 or 60min. The solubility of testosterone in 0.1M-Tris/HCl buffer, pH8.8, was tested by pipetting different amounts of ethanolic solutions of testosterone (0.1–1.3mM), adding a drop of propylene glycol and evaporating the ethanol under N_2. To the residue 1ml of buffer and 80μg of microsomal protein were added; the tubes were shaken for 60min at 37°C, and after cooling to room temperature (22°C) the A_{240} was measured in a Zeiss PMQ II spectrophotometer. The A_{240} was linear with concentration up to 700μM-testosterone, indicating that concentrations above this amount were not completely soluble.

The incubations were terminated by placing the tubes, after stoppering, in a boiling-water bath for 5–10min. The unchanged steroid substrate was quantitatively removed from the aqueous phase by a single extraction with 10ml of water-saturated dichloromethane under these conditions. After a few minutes a portion (0.5ml) of the aqueous phase, which contains the radioactive steroid glucuronide, was transferred to counting vials, 12ml of Bray's (1960) scintillation fluid was added, the mixture shaken vigorously and the radioactivity measured in a Packard liquid-scintillation spectrometer. The glucuronide formed per amount of microsomal protein incubated was calculated from the specific radioactivity in the aqueous phase. Specific activity of the enzyme is expressed as nmol of glucuronide formed/min per mg of protein. In control incubations UDP-glucuronic acid was omitted; all incubations were carried out in duplicate. The variation coefficient [(S.D. $\times$ 100)/$\bar{x}$] of the assay method was 6 and 4% for the assay of oestrone and testosterone glucuronyltransferases respectively. The extraction by dichloromethane enables quantitative recovery of the unchanged steroid substrate, which can be re-used after purification by paper or column chromatography; the aqueous phase is a source of steroid glucuronide.

Treatment of microsomal preparations with detergents

The effects of sodium deoxycholate, Triton X-100, sodium dodecyl sulphate and Lubrol WX on microsomal oestrone and testosterone glucuronyltransferase were investigated by exposing the microsomal preparation to different concentrations (0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10mg of detergent/mg of microsomal protein) of the detergents for 30min at 2–4°C, followed by incubation with the substrates as described under 'Assay of UDP-glucuronyltransferase activities'. Microsomal preparation not treated with detergent served as controls.

Treatment of rats with phenobarbital, 3-methylcholanthrene, oestradiol-17β, testosterone, actinomycin D and cycloheximide for induction studies

In short-term studies, the animals were killed after a single intravenous injection of 50 or 100mg of phenobarbital per kg body wt. at different times up to 24h after drug administration. In long-term studies the animals were killed on different days up to 10 days after daily intraperitoneal injections of 100mg of phenobarbital per kg body wt. The control group of animals received the same volume of vehicle (sterilized 0.9% NaCl) that was used to dissolve the drug. Experiments studying the effect of 3-methylcholanthrene on the induction of steroid glucuronyltransferases were conducted for up to 9 days (long-term studies). The dose of the carcinogen used was 40mg/kg body wt. and was administered as a single injection; the animals were killed every consecutive day. Animals that served as controls received the corresponding amount of olive oil, the vehicle for 3-methylcholanthrene. Actinomycin D and cycloheximide were dissolved in sterile 0.9% NaCl; the doses were 100μg/kg body wt. and 0.5mg/kg body wt. respectively. Both drugs were administered intraperitoneally twice a day, half of the dose each time. The doses chosen for

oestradiol-17β and testosterone were empirical and were 200μg and 10mg/kg body wt. respectively. The steroids were dissolved in olive oil and injected intramuscularly once a day in the morning. The ovariectomized rats received oestradiol-17β and the castrated rats testosterone. Ovariectomized or castrated animals injected with olive oil alone were used as controls. The livers of three animals were pooled and steroid glucuronyltransferase activities were tested in the microsomal preparation after treatment with Lubrol WX for 30min. All results are means$\pm$1 S.D. with number of animals per determination in parentheses. The data were analysed by Student's t test; a P value less than 0.05 was considered to be statistically significant.

Results and Discussion

Separation and quantification of the glucuronides of oestrone, oestradiol-17β, oestriol and testosterone formed during incubation with microsomal preparations from liver, kidney and intestine

The procedure of chromatography of steroid glucuronides described in the Experimental section achieves adequate separation from the incubated steroids and their glucuronides and is suitable as a purification method for the purpose of identification. The rates of glucuronide formation with oestrone, oestradiol-17β, oestriol and testosterone are shown in Table 1. Control incubations from which UDP-glucuronic acid was omitted were simultaneously carried out with each steroid as substrate. The results of the screening study suggest the existence of at least two different enzyme activities in the microsomal fraction of the liver of male and female rats; one, the C-3 glucuronyltransferase, conjugating the C-3 hydroxyl group of oestrone and oestradiol-17β, and the other, the C-17 glucuronyltransferase, conjugating the C-17 hydroxyl group of oestradiol-17β and testosterone.

The microsomal fraction from the liver possesses higher glucuronyltransferase activity than that from kidney and intestine. Oestrone and oestradiol-17β are glucuronidated to a larger extent than is oestriol by either liver or intestine. The site of conjugation of glucuronic acid to the oestrogens is predominantly at C-3. A marked difference in glucuronidating activity is discernible between the male and female rat, in that the microsomal preparation from the liver of female rats is more active

Table 1. *Formation of glucuronides of oestrone, oestradiol-17β and testosterone by microsomal preparations from liver, kidney and intestine of the rat*

Tubes containing 2μCi of 4-^{14}C-labelled steroid, corresponding to 20nmol of non-labelled compound, 2mM-UDP-glucuronic acid and 4mM-Mg^{2+} were incubated with 100–200μg of microsomal protein for 60min at 37°C in 0.1M-Tris/HCl buffer, pH7.4. After incubation butan-1-ol extracts of the incubation mixture were prepared and chromatographed as described under 'Extraction and chromatography of the glucuronides'. The amount of glucuronide formed was calculated from the radioactivity eluted from the paper after two chromatographic steps.

Steroid incubated	Tissues	Product formed	Apparent rates of glucuronidation (nmol/h per mg of protein) Male	Female
Oestrone	Liver	Oestrone 3-glucuronide	3.7	39.1
	Kidney	Oestrone 3-glucuronide	0	0.4
	Intestine	Oestrone 3-glucuronide	2.1	0.5
Oestradiol-17β	Liver	Oestradiol-17β 3-glucuronide	5.2	23.6
		Oestradiol-17β glucuronide	8.6	18.8
	Kidney	Oestradiol-17β 3-glucuronide	3.6	5.3
	Intestine	Oestradiol-17β 3-glucuronide	3.5	2.7
Oestriol	Liver	Oestriol 3-glucuronide	0.4	3.6
		Oestriol 3,16(17)-diglucuronide*	0.8	2.4
	Kidney	Oestriol 3-glucuronide	0.5	2.2
		Oestriol 3,16(17)-diglucuronide*	0.5	2.2
	Intestine	Oestriol 3-glucuronide	10.9	1.1
		Oestriol 3,16(17)-diglucuronide*	0.4	0.9
Testosterone	Liver	Testosterone 17β-glucuronide	9.6	50.1
	Kidney	None		
	Intestine	None		

* The identity of this peak of radioactivity as a diglucuronide was deduced from its inability to move from the start after prolonged (50–60h) chromatography. On elution and incubation at 37°C for 48h in 0.1M-sodium acetate buffer, pH5.0, with β-glucuronidase, oestriol was released. The evidence that the product was a diglucuronide must be considered as tentative.

than that from male rats when oestrone and oestradiol-17β are used as substrates. With oestriol as substrate the specific activity of the enzyme from the intestine of male rats was about 10 times more active than that from female rats. Male and female rats also showed an apparent difference in glucuronidating activity when the substrate was testosterone. This difference is attributable to assay with subsaturating amounts of testosterone. When saturating amounts were used, the differences tended to disappear. This reasoning does not apply to the three oestrogens, since the amounts used in this experiment happened to be close to saturating concentrations. The microsomal preparations from kidney and intestine of both sexes do not possess any transferase activity toward testosterone, which probably indicates that the liver is the main site of glucuronidation of testosterone in the rat.

With cortisol, cortisone, corticosterone and aldosterone as substrates, the amounts of glucuronide formed were less than 0.3% of the total radioactivity incubated. In another series of incubations the ring A-reduced derivatives of cortisol and cortisone were used as substrates, as it is known that these compounds are better substrates for the glucuronyltransferase (Stevens *et al.*, 1961). After chromatography of the incubation extract on paper, small peaks of highly polar radioactive material were obtained, which did not correspond to authentic glucuronides that were chromatographed simultaneously as standards. Varying the incubation conditions (e.g. for pH and time) gave no evidence of glucuronidation of tetrahydrocortisol or tetrahydrocortisone. Miller *et al.* (1973) reported that microsomal preparations from liver of male rat, when treated with Triton X-100, conjugated tetrahydrocortisone. It is possible that the detergent may have exposed enzyme sites for glucuronidation of the ring-A-reduced glucocorticoids. Whether rat liver does indeed form glucuronides of tetrahydro compounds must await isolation and identification of the products of the transferase reaction.

Effects of detergents on microsomal glucuronyltransferase

Detergents are known to enhance the enzyme activities of microsomal preparations (Winsnes, 1973). The activity of microsomal UDP-glucuronyltransferase with several endogenous and non-endogenous compounds as substrates can also be stimulated by detergents (Lueders & Kuff, 1967; Winsnes, 1969; Bock *et al.*, 1973; Jacobson *et al.*, 1975; Lucier *et al.*, 1975). Since differences in activation with different detergents exist, it was essential to know with which detergent and at which concentration maximum activation of enzyme activity could be obtained. From preliminary experiments with untreated microsomal preparation, optimum assay conditions (pH, time, amount of protein and substrate concentration) were established. The effect of treatment of microsomal preparation with four different detergents with oestrone as substrate was investigated. Sodium deoxycholate has no effect on enzyme activity up to 1.0mg/mg of protein. Triton X-100, a commonly used detergent, at a concentration of 0.5mg/mg of protein stimulates the activity 1.5 times compared with microsomal preparations not treated with the detergent. Sodium dodecyl sulphate stimulates the activity 1.34-fold at 0.05mg/mg of protein. Lubrol WX at 0.1mg/mg of protein stimulates the activity maximally 2.45-fold. All detergents were inhibitory above a concentration of 1mg/mg of protein. Varying the pH of the medium in which detergent treatment was carried out, from pH7.4 to 10.0 (0.1M-Tris/HCl buffer, pH7.4–9.0, and 0.1M-glycine/NaOH buffer, pH9.6–10.6) did not exert any influence on enzyme activity. At pH10.6 a decrease in activity was observed. Testosterone glucuronyltransferase was stimulated 3.7-fold by treatment with 0.1mg of Lubrol WX/mg of protein. Thus these results confirm that detergents may activate or inhibit UDP-glucuronyltransferases; the activation depends on the kind of substrate and on the amount of detergents used. All incubations were later carried out with microsomal preparations treated with 0.1mg of Lubrol WX/mg of protein unless otherwise mentioned.

Studies with oestrone as substrate

Effect of duration of incubation, amount of microsomal protein and pH on oestrone glucuronyltransferase activity. The rate of formation of oestrone glucuronide by microsomal preparations treated with Lubrol WX is zero-order with respect to time up to 60min, which indicates that the enzyme reaction is not dependent on the concentration of either oestrone or UDP-glucuronic acid used in this experiment. The activity of the enzyme is linear up to 160μg of microsomal protein. Maximum activity of the untreated microsomal preparation is attained between pH8.4 and 9.6 in 0.1M-Tris/HCl buffer. When the preparation is treated with 0.1mg of Lubrol WX/mg of microsomal protein for 30min at 2–4°C and then incubated, the rates of glucuronide formation increase, and at pH8.4–8.8 enzyme activity is maximally stimulated (2.3-fold over the activity of the untreated preparation). Thus treatment with Lubrol WX does not significantly affect the optimum pH range of the transferase activity.

Effect of Lubrol WX on the kinetic parameters of oestrone glucuronyltransferase. Having established linearity of glucuronide formation with respect to time and amount of protein and selecting the appropriate pH, we decided to investigate the

influence of Lubrol WX on the kinetics of the transferase reaction. For this purpose the concentration of oestrone was varied from 3.3 to 33 μM while the concentration of UDP-glucuronic acid was held constant at 1 mM. Two sets of incubations were carried out, one with untreated microsomal preparation and the other treated with 0.1 mg of Lubrol WX/mg of protein. The $V_{max.}$ values in the absence and in the presence of Lubrol WX, calculated from the Lineweaver & Burk (1934) plot, were 1.9 and 5.4 nmol/30 min per 80 μg of protein respectively. The apparent K_m values for oestrone with treated and untreated microsomal preparations were 11.3 and 13.2 μM respectively. The Hill (1910) plots were linear over the range of oestrone concentrations used; at high concentrations a slight deviation from linearity was observed. The Hill coefficient, h, was calculated to be 0.8 in both cases. This indicates that neither the affinity of the enzyme toward oestrone is affected, nor are additional sites on the enzyme exposed to which oestrone can be bound as a result of treatment with the detergents. One explanation for the increase in $V_{max.}$ values could be that the detergent disperses the endoplasmic-reticulum membranes, facilitating rapid binding of the substrates, leading ultimately to an increase in velocity. It is possible that the detergent also reacts with the substrates during the incubation period to form complexes that are better substrates for the enzyme. On the other hand the increase in activity may be the result of both processes. Since enzyme activity does not depend on the presence of the detergent, the activation should be considered as not essential.

Dependence of glucuronyltransferase activity on the concentration of oestrone and UDP-glucuronic acid. The above experiments were carried out at a fixed concentration (1 mM) of one substrate, UDP-glucuronic acid. As UDP-glucuronyltransferases catalyse a reaction between two substrates, the K_m and $V_{max.}$ values obtained should be considered as apparent. To find an estimate of the real K_m and $V_{max.}$ values (Segel, 1975), a series of incubations was carried out, in which one substrate was varied and different fixed concentrations of the second substrate were used. The data were plotted by the method of Lineweaver & Burk (1934). Different fixed concentrations of UDP-glucuronic acid from 0.5 to 5.0 mM at various concentrations of oestrone, with micro-

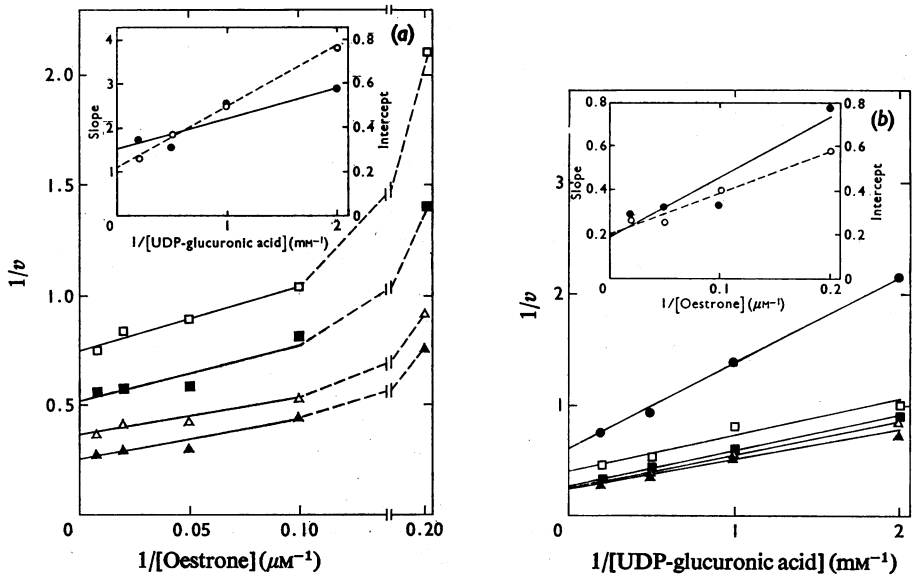

Fig. 1. *Initial-velocity studies with rat liver microsomal oestrone glucuronyltransferase*

The microsomal fraction treated with Lubrol WX was used for incubations. The protein concentration was 100 μg/ml. Incubation and extraction were carried out as described in the Experimental section; v is the rate of oestrone glucuronide formation expressed as nmol/60 min per 100 μg of protein. (*a*) Double-reciprocal plots of the concentrations of oestrone against the rate of formation of oestrone glucuronide. Four different concentrations of UDP-glucuronic acid were used: □, 0.5 mM; ■, 1.0 mM; △, 2.0 mM; ▲, 5.0 mM. Inset: replot of the (1/v)-axis intercepts (○) and slopes (●) against 1/[UDP-glucuronic acid]. (*b*) Double-reciprocal plots of the concentration of UDP-glucuronic acid against the rate of formation of oestrone glucuronide. The oestrone concentrations were: ●, 5.0 μM; □, 10 μM; ■, 20 μM; △, 50 μM; ▲, 120 μM. Inset: replot of the (1/v)-axis intercepts (○) and slopes (●) against 1/[oestrone]. The straight lines for the linear part of the curves were calculated by an unweighted linear-regression program.

somal preparation treated with Lubrol WX, yielded a family of lines that appear parallel, but in effect intersect below the abscissa (Fig. 1*a*). At concentrations lower than 5 μM-oestrone, the points deviate from a straight line, indicating a possible co-operativity caused by the steroid substrate. A replot of the slopes of 1/[oestrone] versus 1/[UDP-glucuronic acid] yielded a $K_{\text{UDP-glucuronic acid}}$ of 460 μM (Fig. 1*a*, inset). A $V_{max.}$ value of 4.6 nmol/30 min per 100 μg of protein was obtained from replots of $(1/V_{max.app.})$ versus 1/[UDP-glucuronic acid]. $\alpha K_{\text{UDP-glucuronic acid}}$ obtained from Fig. 1(*a*, inset) was calculated to be 1.3 mM. From the values of $\alpha K_{\text{UDP-glucuronic acid}}$ and $K_{\text{UDP-glucuronic acid}}$, the factor α was calculated to be 2.8. Since the factor α is greater than unity, the result suggests that increasing concentrations of UDP-glucuronic acid decrease the affinity of the enzyme for oestrone (Segel, 1975). When a Lineweaver–Burk plot of various concentrations of UDP-glucuronic acid against the rates of glucuronide formation at fixed concentrations of oestrone was drawn, a family of lines was obtained with an intersection point above the abscissa (Fig. 1*b*). From a replot of slope against the reciprocal of oestrone concentration, K_{oestrone} was calculated to be 15.5 μM (Fig. 1*b*, inset). The $V_{max.}$ value calculated from Fig. 1(*b*, inset) was 4.7 nmol/30 min per 100 μg of protein; $\alpha K_{\text{oestrone}}$ was 9.3 μM. The factor α was 0.6, suggesting that increasing concentrations of oestrone increase the affinity of the enzyme toward UDP-glucuronic acid (Segel, 1975). Since the straight lines of the initial-velocity studies (Figs. 1*a* and 1*b*) intersect on the left-hand side of the ordinate, a sequential mechanism of the glucuronidation reaction could be postulated, i.e. both substrates must add to the enzyme before a product is released. In view of these results it may be speculated that the enzyme *in vivo* is not saturated, either by oestrone or by UDP-glucuronic acid. Assuming the concentration of oestrone to be in the nanomolar range (from studies in humans) and the concentration of UDP-glucuronic acid to be 120 μM as shown by Zhivkov (1970), the enzyme is capable of increasing glucuronide synthesis even when the concentrations of both substrates increase.

Effect of steroid and non-steroid substrates on the glucuronidation of oestrone. Screening experiments showed that oestradiol-17β and testosterone were glucuronidated to appreciable extents, whereas oestriol was comparatively poorly glucuronidated. Hence it was decided to investigate the influence of these endogenous steroids on the glucuronidation of oestrone in order to gain some knowledge about the specificity of oestrone glucuronyltransferase. In the presence of 40 μM-oestradiol-17β or 40 μM-testosterone (Fig. 2*a*) the initial velocities and the maximum velocities are decreased. The inhibition in both instances is of a mixed type, being predominantly competitive with oestradiol-17β and non-competitive with testosterone as inhibitor. Oestriol at two concentrations, 20 and 40 μM, had no effect on the glucuronidation of oestrone.

Bilirubin has often been used as a substrate for liver microsomal UDP-glucuronyltransferase (Van Roy & Heirwegh, 1968; Mulder, 1972). Since it is known that certain steroids can inhibit the glucuronidation of bilirubin *in vivo* (Arias *et al.*, 1964), it was decided to find out what effect bilirubin would have on oestrone glucuronyltransferase. Fig. 2(*b*) shows that 60 μM-bilirubin inhibited the glucuronidation of oestrone non-competitively, suggesting that these two endogenous substrates are

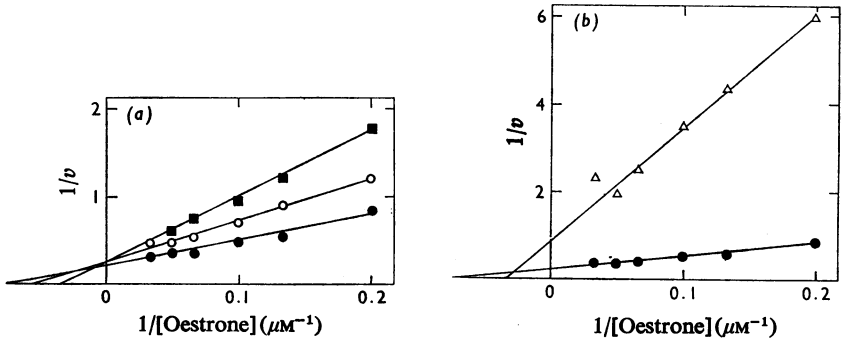

Fig. 2. *Effect of oestradiol-17β, testosterone and bilirubin on oestrone glucuronyltransferase*

Initial-velocity studies with increasing concentrations of oestrone and 1.0 mM-UDP-glucuronic acid. ●, Control; (*a*) ○, in the presence of 40 μM-testosterone; ■, in the presence of 40 μM-oestradiol-17β; (*b*) △, in the presence of 60 μM-bilirubin. For details see the Experimental section; *v* is the rate of oestrone glucuronide formation expressed as nmol/60 min per 100 μg of protein. The protein concentration in this and the following Figures was 100 μg/ml unless otherwise mentioned.

glucuronidated by separate enzymes. Several investigators have used *p*-nitrophenol as substrate for the rat liver microsomal glucuronyltransferase. In order to study whether the same enzyme conjugates *p*-nitrophenol and oestrone, the influence of this 'foreign' substrate on oestrone glucuronidation was studied. *p*-Nitrophenol at concentrations of 60 and 200 μM has no influence on the enzyme conjugating oestrone, over the concentration range of oestrone investigated (3–33 μM). This experiment was carried out with microsomal preparations treated and untreated with Lubrol WX; from both preparations identical results were obtained. Thus in spite of the similarity between ring A of oestrone and *p*-nitrophenol, both substrates are probably glucuronidated by different enzymes. Lucier *et al.* (1975) also reached a similar conclusion from their studies on the effect of treatment with drugs on *p*-nitrophenol and steroid glucuronidation.

These results suggest that UDP-glucuronyltransferases from rat liver possess a considerable degree of specificity toward steroids as substrates. Although oestradiol-17β increases the apparent $K_{oestrone}$ value about twofold, it is quite possible that the same enzyme glucuronidates oestrone as well as oestradiol-17β at C-3. On the other hand, oestradiol-17β is also glucuronidated by microsomal preparation at C-17, suggesting the existence of a different enzyme; this enzyme may also accept testosterone as substrate, forming testosterone 17β-glucuronide as product. In spite of the existence of two different enzymes, the formation of a diglucuronide, e.g. oestradiol 3,17β-diglucuronide, could not be detected, which could mean that the monoglucuronide, oestradiol 17β-glucuronide, is not a substrate for the C-3 transferase and that oestradiol-17β 3-(mono)glucuronide is not a substrate for the C-17 transferase. Direct evidence supporting this hypothesis is, however, lacking.

Localization of oestrone glucuronyltransferase activity in microsomal subfractions. We had earlier shown that the oestriol 16α-glucuronyltransferase from human liver microsomal preparation was localized predominantly in the smooth endoplasmic reticulum (Rao *et al.*, 1970*a*). In the present studies rat liver microsomal preparation was fractionated by centrifugation on a discontinuous sucrose gradient containing 15mM-CsCl, by the method described by Dallner *et al.* (1966). Oestrone glucuronyltransferase activity was tested in the subfractions with and without treatment with Lubrol WX (Table 2). The enzyme activity in the unfractionated microsomal preparation is stimulated 2.5-fold after treatment with Lubrol WX, which confirms the results from previous experiments. The smooth endoplasmic reticulum alone shows slightly higher activity than the total untreated microsomal fraction and its activity is further stimulated 2.1-fold after treatment with Lubrol WX. The rough endoplasmic reticulum possesses minimal activity, which cannot be stimulated further with Lubrol WX. These results clearly show that oestrone glucuronyltransferase activity is localized mainly in the smooth endoplasmic reticulum.

Table 2. *Fractionation of a microsomal preparation from liver on a discontinuous sucrose gradient containing* 15mM-*CsCl and localization of oestrone glucuronyltransferase activity in the microsomal subfractions*

Sucrose gradients containing CsCl were prepared as described by Dallner *et al.* (1966). The microsomal subfractions were treated with 0.1 mg of Lubrol WX/mg of protein for 30min at 2–4°C. Incubation of the subfractions with oestrone and UDP-glucuronic acid were done as described under 'Assay of glucuronyltransferase activities'.

Cell fraction	Oestrone glucuronide formed (nmol/min per mg of protein)	
	Untreated	Treated with Lubrol WX
Unfractionated microsomal fraction	0.70	1.72
Smooth endoplasmic reticulum	0.91	1.98
Rough endoplasmic reticulum	0.19	0.20

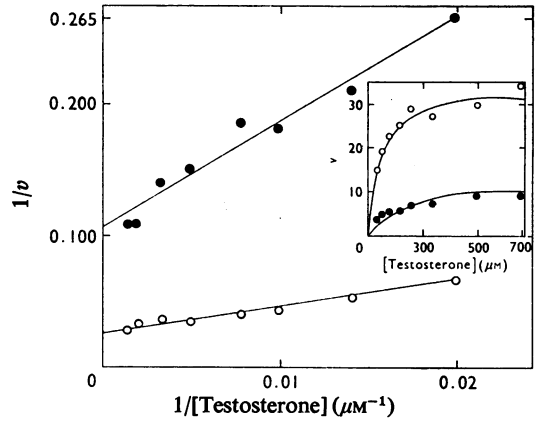

Fig. 3. *Initial-velocity studies with testosterone glucuronyltransferase in the absence and presence of Lubrol WX*

Microsomal preparations from rat liver treated and not treated with Lubrol WX were incubated with increasing concentrations of testosterone at a constant concentration (2mM) of UDP-glucuronic acid; *v* is the rate of testosterone glucuronide formation expressed as nmol/60min per 80μg of protein. ●, Activity of microsomal preparation not treated with Lubrol WX; ○, activity obtained after treating microsomal preparation with 0.1 mg of Lubrol WX/mg of protein. Inset shows velocity, *v*, versus concentration of testosterone.

Studies with testosterone as substrate

Dependence of glucuronyltransferase activity on the concentration of testosterone. From preliminary experiments the rate of testosterone glucuronide formation with microsomal preparation not treated with Lubrol WX was linear up to 60min and up to 120μg of microsomal protein. The enzyme exhibits a plateau of maximum activity between pH8.2 and 8.8 in 0.1M-Tris/HCl buffer. It was observed that the enzyme glucuronidating testosterone required larger amounts of substrate than did the oestrone glucuronyltransferase. Therefore the dependence of transferase activity was studied by incubating 100–700μM-testosterone with microsomal preparation untreated and treated with Lubrol WX (Fig. 3). The rate of glucuronidation reaches a plateau at about 300μM in both instances. Lineweaver–Burk (1934) plots of the data give values for apparent $K_{testosterone}$ of 74μM with Lubrol-treated microsomal preparation and 64μM with an untreated preparation; the apparent $V_{max.}$ values were 36 and 9.4nmol/60min per 80μg of protein respectively. The Hill plots were linear and the coefficients, h, calculated from experiments with and without Lubrol WX, gave values of 1.1 and 0.9 respectively. These results are consistent with Lubrol WX increasing the $V_{max.}$ of the reaction without changing the affinity of the enzyme toward testosterone and without unravelling new binding sites on the enzyme.

Studies on induction of steroid glucuronyltransferases

Effect of administration of phenobarbital on oestrone glucuronyltransferase. Administration of phenobarbital is known to induce microsomal enzyme activity catalysing oxidation, reduction and glucuronidation reactions (Conney, 1967). The effect of phenobarbital on the activity of rat liver microsomal transferase with oestrone as substrate was studied (*a*) by a single intravenous injection (short-term study) and (*b*) by daily intraperitoneal injections of the drug (long-term study). In the short-term study the animals were killed 1, 3, 6, 9 and 24h after injection. Administration of a dose of 50mg of phenobarbital/kg body wt. affected neither the liver wet weight nor the specific activity of oestrone glucuronyltransferase over a period of 24h. Therefore experiments were carried out with 100mg of phenobarbital/kg body wt. The liver wet weight did not change, whereas the specific activity of the transferase increased to 110% of the control value after 24h ($P<0.01$). Since it appeared that enzyme activity could be stimulated with the high dose (100mg) of phenobarbital, the influence of long-term administration of the drug was investigated. The liver wet weight increased to a maximum of 147% of control ($P<0.05$) on day 5 and remained the same up to day 10. The specific activity of the enzyme remained about the control value up to 8 days. On day 10 of treatment it increased slightly. These results indicate that prolonged treatment of female rats with phenobarbital does not lead to significant stimulation of oestrone glucuronyltransferase activity. Similar results were also obtained by Jacobson *et al.* (1975) with oestrone as substrate and microsomal preparation from liver of rats given phenobarbital orally.

Effect of administration of 3-methylcholanthrene on oestrone glucuronyltransferase. Unlike phenobarbital, 3-methylcholanthrene, a polycyclic aromatic hydrocarbon, induces a limited number of microsomal enzymes (Conney, 1967) and has been used to stimulate glucuronyltransferases with xenobiotics (Bock *et al.*, 1973; Howland & Burkhalter, 1971), bilirubin (Potrepka & Spratt, 1971) and with thyroxine (Goldstein & Taurog, 1968) as substrates. The effect of 3-methylcholanthrene on steroid (oestrone) glucuronyltransferase, however, is not known and was therefore investigated. After administration of 3-methylcholanthrene intraperitoneally, the liver wet weight increased up to 125% of the control value on day 3 and remained constant until day 9. The specific activity of oestrone glucuronyltransferase increased to 135% of the control value ($P<0.05$) on day 4 of treatment, then varied between 110 and 135% over the rest of the investigation period. This variation could be due to resorption of the carcinogen, as the livers of several animals were covered with a coat of unabsorbed carcinogen. These results indicate that 3-methylcholanthrene leads to a moderate increase in liver weight and specific activity of oestrone glucuronyltransferase.

Effect of ovariectomy and treatment of ovariectomized animals with oestradiol-17β. Induction of microsomal glucuronyltransferases by endogenous compounds should reflect to some extent the control mechanisms to which the enzyme is subjected depending on the concentration of circulating substrates in the blood. For example, does the activity of a particular enzyme increase when the substrate of this enzyme is increased by administration of the substrate to the animal, in the present case, the administration of oestradiol-17β on the activity of oestrogen glucuronyltransferase? And secondly, which effect does withdrawal of oestrogens, e.g. by ovariectomy, have on the enzyme activity in the liver? The following experiments were carried out to obtain answers to these questions.

Sexually mature female rats were ovariectomized as described in the Experimental section and used for experiments 17–19 days after operation. Fig. 4 clearly indicates that the specific activity of oestrone glucuronyltransferase is decreased from a normal (non-operated animals) value of 1.6 to 0.91 ($P<0.05$) 19 days after ovariectomy. If the animals were used 30 days after ovariectomy the microsomal prepara-

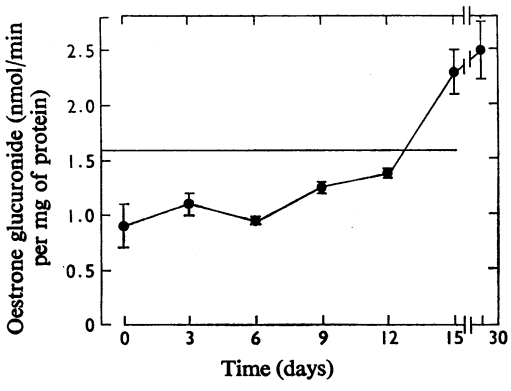

Fig. 4. *Effect of ovariectomy and administration of oestradiol-17β on rat liver microsomal oestrone glucuronyltransferase*

Sexually mature female rats were ovariectomized as described in the Experimental section and used for experimentation 17–19 days after operation. Groups of three animals were injected daily with 200μg of oestradiol-17β/kg body wt.; day zero is the first day of injection. Control animals received the vehicle, olive oil. On the indicated days a group of three animals was killed and the livers were removed and pooled. Microsomal preparations were treated with Lubrol WX, and oestrone glucuronyltransferase activity was measured as described in the Experimental section. The solid line indicates the activity of non-ovariectomized and untreated animals. ●, Specific activity of the enzyme from animals ovariectomized and treated daily with oestradiol-17β; the vertical bars indicate ±1 S.D.

tion had the same activity as on day 19. To investigate the effect of administration of oestradiol-17β on oestrone glucuronyltransferase, ovariectomized animals were injected daily intramuscularly with 200μg of oestradiol-17β/kg body wt. Control animals receiving the vehicle only (olive oil) were killed on day 6. Enzyme activity increased after 6 days of oestradiol-17β treatment (Fig. 4); the specific activity continued to rise over the next 6 days and reached a value of 2.3 on day 15. This is a 2.6-fold increase over the value for ovariectomized non-treated animals ($P<0.01$). The activity appeared to change very little when treatment with oestradiol-17β was continued for another 15 days. Non-ovariectomized animals did not react to oestradiol-17β treatment. The results indicate that ovariectomy leads to a decrease in enzyme activity which can be reversed and even considerably increased by supplementing with oestradiol-17β. Thus it is likely that one factor that governs the activity of oestrone glucuronyltransferase is the amount of circulating oestrogen. The fluctuations in the concentration of oestrogens during the oestrus cycle apparently do not influence the activity of oestrone glucuronyltransferase (solid line in Fig. 4). The increase in activity after prolonged exposure to oestrogens probably points to a homoeostatic function of this enzyme in oestrogen metabolism.

Effects of actinomycin D and cycloheximide on oestradiol-17β-induced increase of oestrone glucuronyltransferase. The increase in activity of oestrone glucuronyltransferase after oestradiol-17β treatment was investigated with actinomycin D and cycloheximide, agents known to inhibit synthesis of RNA and protein respectively. The results in Fig. 5 show that daily intramuscular injections of oestradiol-17β to ovariectomized rats for 15 days increased the activity of oestrone glucuronyltransferase by 110% over the control value ($P<0.01$). When the animals

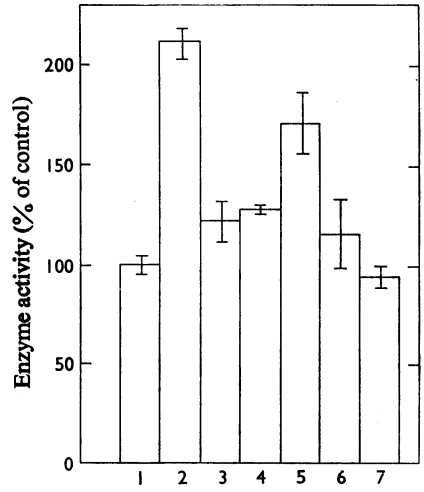

Fig. 5. *Effect of actinomycin D and cycloheximide on oestradiol-17β-induced oestrone glucuronyltransferase of rat liver*

Rats were ovariectomized as described in the Experimental section and were used 19 days after operation. The animals were treated for 15 days as follows: (1) with the vehicle for oestradiol-17β, i.e. olive oil (controls); (2) with 200μg of oestradiol-17β/kg body wt. intramuscularly; (3) with oestradiol-17β plus 100μg of actinomycin D/kg body wt., intraperitoneally; (4) with oestradiol-17β plus 0.5mg of cycloheximide/kg body wt., intraperitoneally; (5) with actinomycin D intraperitoneally; (6) with cycloheximide intraperitoneally; (7) ovariectomized animals, not treated. The animals were killed and the livers removed and pooled. Microsomal preparations were treated with Lubrol WX and oestrone glucuronyltransferase activity was measured as described in the Experimental section. Data are percentages of the value for control rats; the vertical bars indicate ±1 S.D.

were treated with oestradiol-17β and actinomycin D together, the increase in activity observed by oestradiol-17β alone was blocked to a large extent by actinomycin D. Simultaneous injection of cycloheximide and oestradiol-17β also decreased the oestradiol-17β-induced increase in activity. Actinomycin D alone elevated enzyme activity 70% ($P<0.05$) over the control value; with cycloheximide alone the activity was 18% over the control value ($P>0.05$). These results suggest that the increase in oestrone glucuronyltransferase activity observed after administration of oestradiol-17β for 15 days can be blocked to a large extent by RNA- and protein-synthesis inhibitors, indicating that oestradiol-17β probably increases the synthesis of new enzyme. The synthesis occurs after a lag period of 6 days (Fig. 4). The studies also show an apparent induction of oestrone glucuronyltransferase by its substrate oestradiol-17β. The mechanism of induction, however, is unclear. The 1.7-fold increase in enzyme activity observed after administration of actinomycin D alone to ovariectomized rats is difficult to explain in view of its complicated effects.

Effects of administration of oestradiol-17β to ovariectomized rats on testosterone glucuronyltransferase. From the inhibition studies (see Fig. 2) it was likely that the enzymes glucuronidating oestrone and testosterone are not the same. In the present experiment the effect of administering oestradiol-17β to ovariectomized female rats and its influence on testosterone-glucuronidating activity was investigated, to find out whether testosterone glucuronyltransferase could be stimulated by oestradiol-17β. Ovariectomized rats were injected intramuscularly daily with oestradiol-17β, and testosterone glucuronyltransferase activity measured after 3, 6 and 15 days was $6.81\pm0.11(3)$, $5.16\pm0.26(3)$ and $7.5\pm0.9(2)$ respectively. The specific activity of the enzyme from control animals was $6.3\pm0.14(3)$. These results indicate that testosterone glucuronyltransferase is not stimulated in female rats by prolonged treatment of animals with oestradiol-17β, whereas the oestrogen stimulates oestrone glucuronyltransferase 2.6-fold after prolonged treatment. This is additional evidence pointing to the existence of two separate enzymes, one glucuronidating oestrone, which is inducible by the administration of oestradiol-17β, and the other glucuronidating testosterone, which is not affected when the animals were treated in the same way.

Effect of castration and administration of testosterone on testosterone glucuronyltransferase. As it was observed that ovariectomy decreased oestrone glucuronyltransferase activity, it was decided to investigate the influence of castration on microsomal testosterone glucuronyltransferase activity. The specific activity of the enzyme of liver microsomal preparations from normal male rats was $8.65\pm0.65(13)$. This activity is slightly higher than that of liver microsomal fractions from normal female rats [$6.13\pm0.24(10)$; Rao *et al.*, 1976]. The animals were castrated and enzyme activity was measured 19 days after operation; the value was $8.6\pm1.7(3)$. After administration of testosterone the specific activity of the transferase determined 3, 6, 15, 20 and 30 days after treatment was $8.62\pm0.88(n=3)$, $8.85\pm0.3(3)$, $9.88\pm0.6(3)$, $9.5\pm0.18(3)$ and $8.2\pm0.25(2)$. The control value was $9.81\pm1.2(3)$. The differences in the values when compared with the value from control animals were not significant ($P>0.05$). Thus castration has no effect on the enzyme glucuronidating testosterone, nor does supplementing these animals daily with large doses of testosterone lead to an increase in enzyme activity. Therefore the activity of testosterone glucuronyltransferase is not controlled by its own substrate, which distinguishes it as an entity from oestrone glucuronyltransferase, which is sensitive to ovariectomy and to supplementation of oestrogen. Thus the present results support the existence of multiple steroid glucuronyltransferases in the liver of the rat.

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