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STUDIES ON THE PROPERTIES OF AN ENZYME FORMING THE GLUCURONIDE OF PREGNANEDIOL AND THE PATTERN OF DEVELOPMENT OF STEROID GLUCURONYLTRANSFERASES IN RAT LIVER

MICHAEL FUCHS*, GOVIND S. RAO†, MARIE LUISE RAO and HEINZ BREUER
Institut für Klinische Biochemie der Universität Bonn, 53 Bonn-Venusberg, Federal Republic of Germany

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SUMMARY

The microsomal fraction from liver of female rats was found to glucuronidate pregnanediol. Enzyme activity was stimulated by Mg^{2+}, Ca^{2+} and *n*-ethylmaleimide; *p*-chloromercuribenzoate did not affect enzyme activity, while dithioerythritol inhibited the activity 34%. The K_M values for pregnanediol and UDP-glucuronic acid were 0.84 and 60.8 μM, respectively; the mechanism of enzymatic reaction was sequential. Oestrone, etiocholanolone, pregnanolone and 11-deoxytetrahydrocortisone inhibited pregnanediol glucuronyltransferase competitively; bilirubin and phenolphthalein were noncompetitive inhibitors of the enzyme. Testosterone, tetrahydrocortisone, tetrahydrocorticosterone, *p*-nitrophenol and *o*-aminophenol did not affect pregnanediol glucuronidation. The activation energies with pregnanediol, oestrone and testosterone as substrates were 18.0, 11.7 and 12.0 kcal/(K × mol). Glucuronyltransferases conjugating pregnanediol, oestrone, testosterone and bilirubin show different rates of development and reach maximal activity at different times after birth. These results indicate the presence of an enzyme glucuronidating pregnanediol, which is different from those glucuronidating oestrone, testosterone and bilirubin, and provide additional support to the presence of multiple, steroid specific glucuronyltransferases in the liver of the rat.

INTRODUCTION

Biologically active steroid hormones are lipophilic compounds; as a result of metabolism they are rendered water soluble by conjugation with glucuronic acid. The enzyme, catalyzing this reaction, UDP-glucuronyltransferase (EC 2.4.1.17), is present in the microsomal fraction. Although steroid hormones, such as androgens, oestrogens and glucocorticoids, vary in their chemical constitution, the glucuronyltransferase accepts these diverse compounds as substrates. As will be shown in this study, rat liver microsomes glucuronidate also pregnanediol. From competition studies using steroids and nonsteroids and from studies of the activation energies, it appeared that the enzyme glucuronidating pregnanediol is different from those glucuronidating oestrone, testosterone and bilirubin.

The presence of several different enzymes forming glucuronides of different steroid hormones, has been demonstrated in our laboratory from studies with human liver [1, 2], pig kidney [3] and rat liver [4]. Lucier *et al.*[5] has also shown evidence pointing to the existence of more than one glucuronyltransferase. Taking these findings into consideration one would expect different glucuronyltransferases to exhibit different rates of development and different degrees of activity from the day of birth till adulthood. In order to find out whether this presumption could be demonstrated experimentally, we followed the pattern of development of glucuronyltransferase activities in the microsomes of female rat liver a few hours after birth till day 85, using pregnanediol, oestrone, testosterone and bilirubin as substrates. The findings gave additional support to the concept of multiplicity of steroid glucuronyltransferases.

MATERIALS AND METHODS

Materials

[1,2-$^3H_2$]-Pregnanediol (specific radioactivity 48.6 Ci/mmol) was obtained from New England Nuclear Corporation, Boston, U.S.A. [4-^{14}C]-Oestrone (specific radioactivity 58 mCi/mmol), [4-^{14}C]-testosterone (58 mCi/mmol) and uridine-5-[U-^{14}C]-diphosphoglucuronic acid, ammonium salt (225 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks, England. The non-labelled steroids were from Merck, Darmstadt, Germany. Pregnanediol 3-glucuronide was prepared by Dr. Röhle of this institute according to the method described by Hübner *et al.*[6]. UDP-glucuronic acid disodium salt was from Boehringer, Mannheim, Germany. All other reagents were of analytical grade. Female Wistar rats from Ivanovas, Kissleg, Allgäu, Germany were used for all experiments; they were 2–3 months old and weighed 170–210 g. For experiments on the development of the enzyme in the rat liver, newborn female rats and sexually mature rats

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† To whom all correspondence and requests for reprints should be addressed.

from the same generation were used. Young rats were bred from mature female and male animals at the animal house of the department of Pathology. For experiments the animals were randomly selected from 2 or more mothers.

Methods

Preparation of the microsomal fraction from liver homogenate. All procedures concerning the isolation of the microsomal fraction were carried out at 0–4°C. Adult animals were killed by cervical dislocation; the newborn and young animals were decapitated with a pair of scissors. The livers were homogenized in 0.25 M sucrose in 0.1 M Tris–HCl buffer, pH 7.4, using an Ultra-Turrax (Janke and Kunkel KG., Staufen im Breisgau, Germany). Centrifugation procedures to obtain microsomes were carried out as described in [7]. Protein was determined by the method of Lowry *et al.*[8] with bovine serum albumin as standard.

Determination of pregnanediol glucuronyltransferase activity. The method previously described by Rao *et al.*[7] for the determination of oestrone and testosterone glucuronyltransferases was used with some modifications. [1,2-$^3H_2$]-Pregnanediol was mixed with non-labelled pregnanediol in such a manner that 30,000 c.p.m. (0.1 ml of an ethanolic solution) were equivalent to 30 nmol of the steroid, unless otherwise mentioned. Preparation of samples for incubation was done as described in [7]. The buffer used was 0.1 M Tris–HCl, pH 8.3, and contained 10 mM Mg^{2+} and 1 mM UDP-glucuronic acid, unless otherwise indicated. The incubation was started by adding the microsomal suspension; the total vol. was 1.0 ml. Incubations were carried out in duplicate or triplicate; blank values were obtained from incubations in which UDP-glucuronic acid was omitted. The samples were incubated for 20 min at 37°C. The incubation was stopped by adding 10 ml of ice-cold water-saturated methylene dichloride and the contents were rapidly shaken with the help of a Whirlimixer. The tubes were then centrifuged for 30–40 s at 2,000 *g* to obtain a clear separation between the aqueous and organic phase. A quantitative extraction of the unreacted pregnanediol into the organic phase can be achieved by this extraction procedure. A portion (0.5 ml) of the aqueous phase containing pregnanediol glucuronide was pipetted into a scintillation vial, 12 ml Brays' scintillation fluid was added, and the radioactivity was measured in a liquid scintillation spectrometer, Nuclear Chicago, Mark II. The efficiency was 40%; at least 10,000 counts were accumulated to keep the error below 1%. Activity is expressed as nmol pregnanediol glucuronide formed per amount of enzyme (microsomal protein) incubated; S.A. is expressed as nmol glucuronide formed × mg protein^{-1} × h^{-1}. Under the conditions described, the variation coefficient for the enzyme assay was 2.5%. The sensitivity of the assay of pregnanediol glucuronyltransferase was determined by taking twice the counts of the control incubation and was calculated to be 0.03 nmol pregnanediol glucuronide formed × mg protein^{-1} × h^{-1}. Oestrone and testosterone glucuronyltransferase activities were determined by the method described by Rao *et al.*[7], bilirubin glucuronyltransferase activity by the method of Mulder[9].

Identification of pregnanediol glucuronide. For the purpose of characterization of the radioactive product formed, incubations were carried out using two isotopes. To incubation tubes containing 100 μM pregnanediol and 0.5 mM UDP-glucuronic acid, were added: (I) 0.5 μCi [1,2-$^3H_2$]-pregnanediol, (II) 1.0 μCi [U-^{14}C]-UDP-glucuronic acid and (III) 0.5 μCi [1,2-$^3H_2$]-pregnanediol and 1.0 μCi [U-^{14}C]-UDP-glucuronic acid. Each sample contained 10 mM $MgCl_2$; the total vol. was 2.0 ml. The buffer was 0.1 M Tris–HCl, pH 8.3; the reaction was started by adding the microsomal suspension equivalent to 100 μg protein. The incubation was carried out at 37°C and lasted for 2 h. The reaction was stopped by placing the tubes in ice–water. Sodium chloride was added and the contents were extracted 3 times with 2 ml of water-saturated butan-1-ol. The extracts were combined, evaporated to dryness, the residue was taken up in chloroform–methanol (3:7, v/v) and applied onto a thin layer plate (DC-Alufolien, Kieselgel, 60 F 254, E. Merck, Darmstadt, Germany). Pregnanediol and pregnanediol glucuronide were applied as standards. The thin layer plate was developed in chloroform–methanol (3:7, v/v). The standards were stained by spraying a solution of antimony trichloride in chloroform. The chromatograms were scanned in a Berthold–Frieseke scanner. The peaks of radioactivity were eluted from the thin layer plate with 5 ml chloroform–methanol (3:7, v/v). The organic solution was evaporated and the residue was taken up in 0.5 ml methanol. This solution was oxidized in an oxidizer (Oxymat, Ja 104, Deutsche Intertechnik, Mainz, Germany) and the samples were mixed with 12 ml of a toluene based scintillation mixture according to the specification given by the Deutsche Intertechnik and counted in a liquid scintillation spectrometer. Controls for elution of radioactivity from thin layer plates, for oxidation and for counting of radioactivity were carried out and the results were corrected to 100%.

RESULTS AND DISCUSSION

Identification of the enzymatic product, pregnanediol glucuronide. The results of the incubations of [1,2-$^3H_2$]-pregnanediol and [U-^{14}C]-UDP-glucuronic acid with rat liver microsomes are depicted as thin layer chromatogram scans in Fig. 1. The chromatogram shows peaks of radioactivity of both substrates (pregnanediol and UDP-glucuronic acid) and of pregnanediol glucuronide, since butan-1-ol extracts the substrates and the product. Line I shows the separation of [1,2-$^3H_2$]-pregnanediol and [1,2-$^3H_2$]-pregnanediol glucuronide; line II that of pregnanediol [U-^{14}C]-glucuronide and [U-^{14}C]-UDP-glucuronic

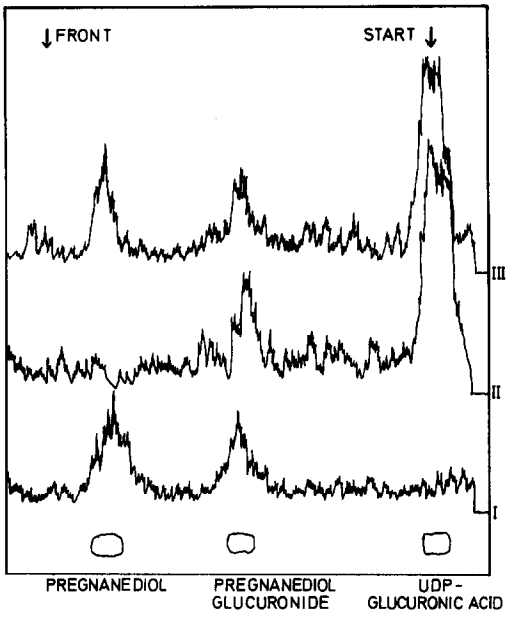

Fig. 1. Thin layer chromatogram of the product of the enzymatic reaction. In three parallel experiments liver microsomes from female rats were incubated with 100 μM non-labelled pregnanediol, 0.5 mM non-labelled UDP-glucuronic acid, 10 mM $MgCl_2$, 100 μg of microsomal protein in 0.1 M Tris–HCl buffer, pH 8.3, for 2 h at 37°C; the total vol. was 2.0 ml. In addition, incubation (I) contained 0.5 μCi [1,2-$^3H_2$]-pregnanediol, incubation (II), 1.0 μCi [U-^{14}C]-UDP-glucuronic acid and incubation (III), 0.5 μCi [1,2-$^3H_2$]-pregnanediol and 1.0 μCi [U-^{14}C]-UDP-glucuronic acid. Extraction of the incubation mixtures and chromatography on thin layer plates were done as described in the text under "Methods".

acid and line III that of [1,2-$^3H_2$]-pregnanediol [U-^{14}C]-glucuronide and the two radioactive substrates. The radioactive pregnanediol glucuronide corresponds in each case to the authentic standard. Oxidation of the eluate of [1,2-$^3H_2$]-pregnanediol [U-^{14}C]-glucuronide of line III gave an isotope ratio of 1.1:1 for ^{3}H and ^{14}C. This agrees with the expected isotope ratio of 1:1 for the monomolecular reaction between pregnanediol and UDP-glucuronic acid, catalyzed by the liver microsomes. These results show that the product of the enzymatic reaction is pregnanediol glucuronide. On elution of [1,2-$^3H_2$]-pregnanediol glucuronide and hydrolysis with β-glucuronidase in 0.1 M acetate buffer, pH 5.0, the substrate pregnanediol could be identified.

Dependence of pregnanediol glucuronide formation on the amount of protein, pH and time. Formation of pregnanediol glucuronide was linear up to 160 μg of microsomal protein; the pH optimum was 8.3 in 0.1 M Tris–HCl buffer. Tris–maleate and glycine–NaOH buffers gave lower enzyme activities. The rate of glucuronide formation at pH 8.3 and at 37°C using 20–100 μg microsomal protein was linear up to 120 min of incubation.

Effect of cations, sulphydryl reagents, ATP and UDP. Incorporation of Mg^{2+} (1–50 mM) Ca^{2+} (1–5 mM), Fe^{2+} (1–50 mM) and Fe^{3+} (1–10 mM) in the incubations stimulated pregnanediol glucuronyltransferase activity 1.5–2.9 fold; Cd^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} (1–10 mM) were strong inhibitors of enzyme activity. Since 10 mM Mg^{2+} stimulated enzyme activity maximally, it was routinely incorporated in the incubations.

n-Ethylmaleimide (0.01 mM) added to the incubation stimulated pregnanediol glucuronyltransferase activity 1.6 fold; *p*-chloromercuribenzoate in concentrations up to 1.0 mM did not affect enzyme activity. This effect by the two -SH group blocking agents is in contrast to their effects on oestrogen glucuronyltransferase [10], which is inhibited by -SH reagents. The observed moderate stimulation in the present case suggests the nonessential nature of reduced disulfide groups. In fact, when incubations were carried out in the presence of dithioerythritol (0.01–1.0 mM) enzyme activity was reduced by 5–34%.

ATP (0.01–1.0 mM) increased the enzyme activity 1.5–1.7 fold. UDP, which is formed as a result of the glucuronide synthesis had no significant effect at concentrations from 0.01 to 1.0 mM, whereas this uridine nucleotide strongly inhibits oestrogen glucuronyl-

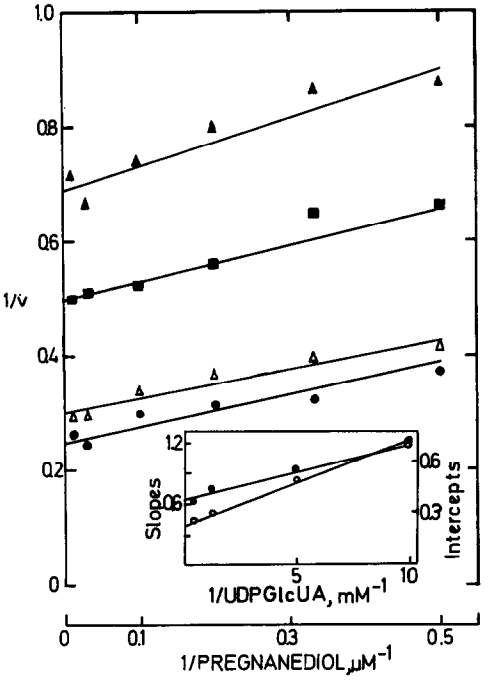

Fig. 2. Double reciprocal plot of pregnanediol glucuronyltransferase. The tubes contained increasing concentrations of non-labelled pregnanediol, 30,000 c.p.m. of [1,2-$^3H_2$]-pregnanediol, increasing fixed concentrations of UDP-glucuronic acid, 10 mM $MgCl_2$, 15 μg microsomal protein and 0.1 M Tris–HCl buffer, pH 8.3; the total vol. of the incubation mixture was 1.0 ml. Duration of incubation was 20 min at 37°C. The concentrations of UDP-glucuronic acid were ●, 2.0 mM; △, 0.8 mM; ■, 0.2 mM and ▲, 0.066 mM; V, is nmol pregnanediol glucuronide × 15 μg protein^{-1} × h^{-1}. The inset shows the slopes and intercepts. The regression lines were calculated by an unweighed program of the method of least squares.

transferase [3, 10, 11] even at lower concentrations. It appears therefore, that the steroid glucuronyltransferase from the microsomes of rat liver is different to the enzyme from intestine and kidney of the pig.

Dependence of glucuronyltransferase activity on pregnanediol and UDP-glucuronic acid concentrations. UDP-glucuronyltransferases catalyze a reaction between two substrates. In order to evaluate the affinities of the two substrates to the enzyme, microsomes were incubated with increasing concentrations of pregnanediol (2–60 μM) and different fixed concentrations of UDP-glucuronic acid. Plotting the initial velocity data according to Lineweaver and Burk[12] resulted in a family of straight lines which intersected below the x-axis (Fig. 2). A secondary plot of the slopes and intercepts is shown in the inset. The reciprocal plot of the results obtained from the same experiment for the other varied ligand, UDP-glucuronic acid, also gave a series of straight lines which intersected on the x-axis. Thus, when pregnanediol or UDP-glucuronic acid were plotted as the varied substrate, the lines intersect on the left side of the y-axis, pointing to a sequential mechanism of the reaction, which means, both substrates must bind to the enzyme, before a product is released. The sequential mechanism appears to be common for steroid glucuronyltransferases from pig kidney [3] and from microsomes of rat liver [4] using oestrone as substrate.

The kinetic constants obtained from these graphical analysis are presented in Table 1. From K_M and αK_M values, factor α [13] can be calculated; this turned out to be 1.08 for pregnanediol, which indicates that differences in the concentrations of pregnanediol do not influence the affinity of the enzyme to UDP-glururonic acid. The value of factor α of 3.22 for UDP-glucuronic acid, is an indication of a decrease in the affinity of the enzyme toward pregnanediol, when UDP-glucuronic acid concentrations are increased. The Hill coefficients [14] of approximately 1 for both substrates indicate that only one molecule of each substrate binds to the enzyme.

Table 1. Kinetic constants of pregnanediol glucuronyltransferase from liver microsomes of the rat

	Kinetic constants			
Substrate	K_M (μM)	αK_M (μM)	Hill coeff.	V_{max} (nmol × 15 μg protein^{-1} × h^{-1})
Pregnanediol	0.84	0.90	1.05	4.12
UDP-glucuronic acid	60.8	196.0	1.33	4.12

For details of incubation refer to "Methods".

Effect of steroid and nonsteroid compounds on pregnanediol glucuronyltransferase. In a previous investigation [4] we showed that liver microsomes from female rats glucuronidated oestrone, oestradiol-17β, oestriol and testosterone. Tetrahydrocortisone and tetrahydrocortisol were not conjugated by liver microsomes. The present studies show that the liver microsomal

Table 2. Effects of steroids and nonsteroids on the formation of pregnanediol glucuronyltransferase

Compound tested	Concentration (μM)	Type of inhibition	$K_{i(app)}$ (μM)
Oestrone	1	Competitive	10.7
	10	Competitive	16.8
Testosterone	10	None	
	20	None	
Etiocholanolone	1	Competitive	1.8
	10	Competitive	5.5
Pregnanolone	1	Competitive	0.72
	10	Competitive	2.34
11-Deoxytetra-hydrocortisone	5	Competitive	4.3
	10	Competitive	3.3
Tetrahydrocortisone	1	None	
	10	None	
Tetrahydrocorti-costerone	1	None	
	10	None	
Bilirubin	10	Noncompetitive	11.5
	20	Noncompetitive	8.1
Phenolphthalein	5	Noncompetitive	7.2
	20	Noncompetitive	29.7
p-Nitrophenol	50	None	
	200	None	
o-Aminophenol	50	None	
	200	None	

Tubes contained increasing concentrations of non-labelled pregnanediol (0.47–2.0 μM), 30,000 c.p.m. [1,2-$^3H_2$]-pregnanediol, 0.2 mM UDP-glucuronic acid, 10 mM $MgCl_2$ and 0.1 M Tris–HCl buffer, pH 8.3, in a total vol. of 1 ml; 10–15 μg of microsomal protein was incubated in the absence and presence of the mentioned steroids and nonsteroid compounds for 10 min at 37°C. Initial velocities were analyzed by Lineweaver–Burk plots. The K_i values were calculated according to Dixon and Webb[15]. The apparent K_M for pregnanediol was 0.52 ± 0.22 μM (n = 8).

fraction also glucuronidates a C-21 steroid, namely pregnanediol. It was therefore essential to find out, whether the above mentioned steroids influence pregnanediol glucuronyltransferase; in addition the effects of bilirubin and 'foreign' substrates, commonly used for the assay of glucuronyltransferase from tissues of different animals, were tested. The results were analyzed by the Lineweaver–Burk plots [12] and are presented in Table 2. From the results the following conclusions may be made: Among the steroids tested, oestrone, etiocholanolone, pregnanolone and 11-deoxytetrahydrocortisone inhibit the pregnanediol glucuronyltransferase competitively. Since the K_i values differ considerably from the K_m value for pregnanediol (0.52 μM), it is unlikely that the tested steroids are glucuronidated by the enzyme conjugating pregnanediol. The competitive inhibition is probably due to the presence of a hydroxyl group at C-3 which facilitates binding of the steroid to the active centre of pregnanediol glucuronyltransferase. This hypothesis fits well with the observation that testosterone, which lacks the C-3 hydroxyl group exerts no influence on pregnanediol glucuronyltransferase. Although tetrahydrocortisone and tetrahydrocorticosterone possess a hydroxyl group at C-3, binding of these steroids to the enzyme molecule is probably obstructed by the presence of a functional group at C-11, with the result that glucuronidation of pregnanediol remains unaltered. The side chain at C-17, e.g. in the case of pregnanolone, 11-deoxytetrahydrocortisone, tetrahydrocorticosterone and tetrahydrocortisone, does not appear to take part, neither in the binding nor in the glucuronidation reaction. For example, tetrahydrocortisone and 11-deoxytetrahydrocortisone, which possess the same side chain in ring D, have different effects on the glucuronidation of pregnanediol (Table 2); one having no influence and the other inhibiting the enzyme competitively. Summarizing these results, it appears that a certain portion of the steroid molecule is involved in the binding to the enzyme, which may lead to the glucuronidation reaction. These results show that the binding site of the glucuronyltransferase possesses high specificity for the steroid aglycone. It was therefore interesting to find out whether this consideration could also be applied to nonsteroids which are substrates for glucuronyltransferases. As can be seen from the results in Table 2, all 4 compounds tested did not bind to the active centre of the enzyme, since none of them inhibited the glucuronidation of pregnanediol competitively. In fact, *p*-nitrophenol and *o*-aminophenol had no influence on the conjugation, whereas bilirubin and phenolphthalein inhibit the steroid glucuronyltransferase non-competitively. Thus, the latter two compounds did not bind to the active site of the steroid glucuronyltransferase. These results strongly suggest multiplicity of glucuronyltransferases, not only with respect to steroid and nonsteroid substrates, but also with respect to differently substituted steroids.

Activation energies of glucuronyltransferases. The aspect of multiplicity of steroid glucuronyltransferases was further investigated by comparing the V_{max} values as a function of temperature, using pregnanediol, oestrone and testosterone as substrates. It is known that differences in the activation energies with different substrates point to the presence of different enzymes, since the activation energies appear to be "more characteristic of the enzyme than of the substrate" [15]. In order to test this assumption, pregnanediol, oestrone and testosterone were incubated with the same preparation of liver microsomes from the female rat at different temperatures. The concentration of pregnanediol was 30 μM and that of oestrone and testosterone was 30 and 600 μM, respectively. These concentrations of the steroids and the respective concentrations of UDP-glucuronic acid lie in the saturating range [7]. The pattern of activity from 5 to 70°C using different steroid substrates is shown in Fig. 3 in the form of an Arrhenius plot. The activation energies with pregnanediol, oestrone and testosterone as substrates were 18.0, 11.7 and 12.0 kcal/(K × mol), respectively; the values were calculated from the linear part of the plot. The temperature optima were 50,

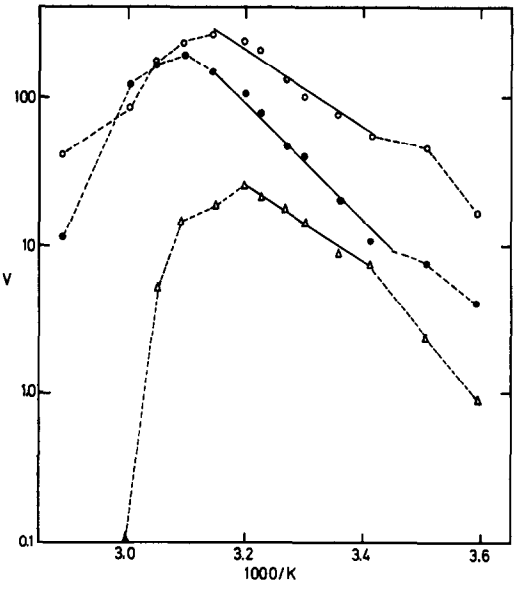

Fig. 3. Arrhenius plot of the glucuronidation of pregnanediol, oestrone and testosterone. For determination of steroid glucuronyltransferase activities incubations were carried out under the following optimal conditions: 30,000 c.p.m. [1,2-$^3H_2$]-pregnanediol corresponding to 30 μM non-labelled steroid, 0.8 mM UDP-glucuronic acid, 30 μg microsomal protein; 100,000 c.p.m. [4-^{14}C]-oestrone corresponding to 30 μM non-labelled oestrone, 1 mM UDP-glucuronic acid and 100 μg microsomal protein and 100,000 c.p.m. [4-^{14}C]-testosterone or 600 μM, 2 mM UDP-glucuronic acid and 100 μg microsomal protein were incubated in 0.1 M Tris–HCl buffer, pH 8.3, containing 10 mM $MgCl_2$ for 20 min at 37°C. Extraction, measurement of radioactivity and calculation of activity are described in the text under "Methods"; *V*, is nmol glucuronide × mg protein^{-1} × h^{-1}; ●, pregnanediol glucuronide; △, oestrone glucuronide and ○, testosterone glucuronide.

40 and 45°C for pregnanediol, oestrone and testosterone, respectively. These results clearly show the dissimilarities between the enzyme of the liver glucuronidating pregnanediol and those glucuronidating oestrone and testosterone.

Pattern of development of pregnanediol, oestrone, testosterone and bilirubin glucuronyltransferases from the postnatal stage to adulthood. The experimental evidence obtained from the above studies point to the existence of different steroid glucuronyltransferases in the liver of the female rat. Another way to demonstrate multiplicity of the enzyme was to follow their pattern of development from the day of birth to the adult stage. The patterns of glucuronyltransferase activities with pregnanediol, oestrone, testosterone and bilirubin as substrates from 30 min after birth till day 85 are shown in Fig. 4. Enzyme activity glucuronidating pregnanediol varies between 0 and 10 from birth till day 33, after which it reaches a plateau on day 40 and remains constant till day 60. Afterwards it increases in a spurt reaching a value of 130 on day 65, and varies between 90 and 115 till day 85. Enzyme activity glucuronidating oestrone exhibits a continuous rise from the day of birth till day 21, on which it has reached its maximum value of 27; later there is no significant change. Enzymic activity glucuronidating bilirubin also shows a continuous rise from birth till day 21 after which the activity remains fairly constant. Enzymic activity glucuronidating testosterone has the highest initial activity as compared to the foregoing three substrates on the day of birth. The initial value of 88 increases to 468 on day 37 and remains between 392 and 442 for the rest of the investigation period. It is obvious from these results, that all four enzymes have different initial activities, show different rates of increase with age and attain maximal activity on different days except for oestrone and bilirubin. These two enzyme activities exhibit an almost similar pattern of development from which one might infer that one enzyme glucuronidates both substrates. However, studies in this laboratory have shown [4] that bilirubin inhibits the conjugation of oestrone non-competitively.

The evidence obtained from the present investigation point to the fact that there are multiple steroid specific glucuronyl transferases, and bilirubin glucuronyltransferase is an entity of its own. The dissimilar pattern of development of glucuronyltransferase activity shows that the enzymes are probably coded by different genes. The correlation of attainment of maximal activity with such factors like changing over from mother's milk to solid food, to the commencement of ovulation or to sexual maturation cannot be drawn at this stage. However, an interesting aspect that arises from this study is the pattern of development of the activities of pregnanediol and bilirubin glucuronyltransferases. Both activities are present at birth; bilirubin glucuronyltransferase activity increases with progressing age, but pregnanediol glucuronyltransferase activity begins to increase only after day 33. From

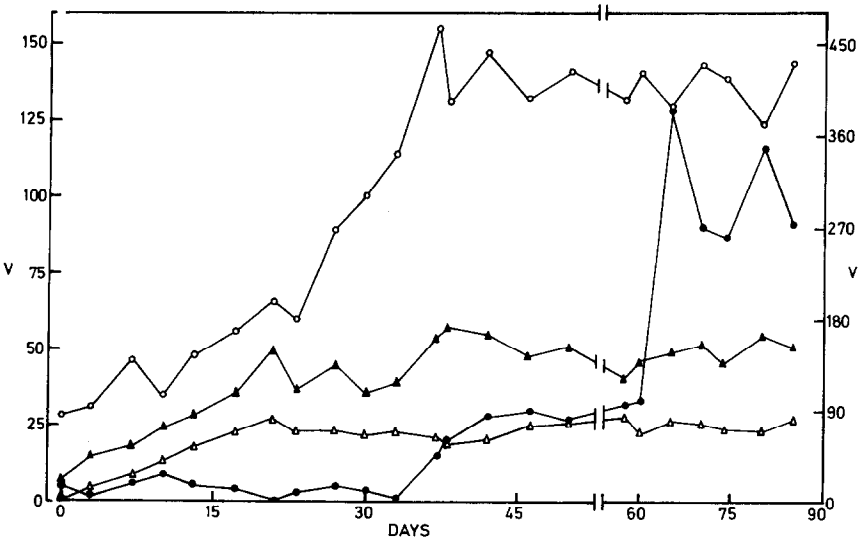

Fig. 4. Pattern of development of pregnanediol, oestrone, testosterone and bilirubin glucuronyltransferases in the liver of the female rat 30 min pospartum till adulthood. Incubations with pregnanediol, oestrone and testosterone as substrates were carried out as described in the legend to Fig. 3. With bilirubin as substrate, enzyme activity was determined according to the method described by Mulder[9]. *V*, is nmol glucuronide × mg protein^{-1} × h^{-1}. The values on the left ordinate show the activities for pregnanediol, oestrone and bilirubin glucuronyltransferases and on the right ordinate the activity for testosterone glucuronyltransferase. For each point the livers from 3–4 animals were combined and a microsomal fraction was isolated; incubations were carried out in triplicate. The sensitivities of pregnanediol, oestrone and testosterone glucuronyltransferase assays were 0.03, 0.0003, 0.066 nmol × mg protein^{-1} × h^{-1}. ●, Pregnanediol glucuronide; △, oestrone glucuronide; ○, testosterone glucuronide and ▲, bilirubin glucuronide.

studies in humans it is known that pregnanediol, which is present in the milk of lactating mothers, inhibits bilirubin glucuronidation, leading to icterus in the first few days of life [16]. Thus, one speculation could be made: if abnormal amounts of pregnanediol are transferred from the mother to the young after birth and are not adequately glucuronidated because of the low activity of the enzyme, pregnanediol begins to accumulate and inhibits bilirubin glucuronyltransferase. Since the inhibition is non-competitive, even subnormal amounts of pregnanediol could interfer with proper elimination of bilirubin.

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