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INVESTIGATIONS ON THE KINETIC PROPERTIES OF ESTRONE GLUCURONYLTRANSFERASE FROM PIG KIDNEY

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Summary

The microsomal fraction of the pig kidney catalyzes the glucuronidation of estrone in the presence of UDP-glucuronic acid. This bireactant system exhibits a sequential type of reaction mechanism. Increasing concentrations of either substrate increase the affinity of the enzyme for the other substrate. The Hill coefficient, n, was calculated to be 1.0 for both estrone and UDP-glucuronic acid. The K_{estrone} and $K_{\text{UDP-glucuronic acid}}$ are 6.6 μM and 254 μM, respectively. The estrone glucuronyltransferase (UDPglucuronate : 17β-oestradiol 3-glucuronosyltransferase, EC 2.4.1.59) exhibits high substrate specificity in that it is inhibited noncompetitively by estradiol-17β, estradiol-17α, estriol, testosterone, phenolphthalein and bilirubin; *p*-nitrophenol and *o*-aminophenol do not inhibit the glucuronidation of estrone. Mg^{2+} and Ca^{2+} were found to be nonessential activators. One of the two products of the reaction, estrone glucuronide, inhibits the enzyme competitively in the presence of increasing concentrations of UDP-glucuronic acid. The other product of the reaction, UDP, inhibits the enzyme noncompetitively with varying estrone concentrations and uncompetitively with varying UDP-glucuronic acid concentrations. Under incubation conditions for the glucuronidation of estrone, the enzyme catalyzes the reverse reaction with estrone glucuronide and UDP as reactants to an extent of about 0.4% of the forward reaction; this reverse reaction is also of a sequential type.

Introduction

Estrogen glucuronyltransferase (UDPglucuronate:17β-oestradiol 3-glucuronosyltransferase, EC 2.4.1.59) from pig intestine [1,2] catalyzes the transfer of glucuronic acid to the phenolic hydroxyl group of estradiol-17β and estrone. The enzyme is tightly bound to the membranes of the endoplasmic reticulum, but can be obtained in a nonsedimentable form by treating with sodium dodecyl sulfate [2]. Investigations in this laboratory have shown that treatment of

rat liver microsomes with detergents, such as Lubrol WX [3] increases the V of the reaction, but does not affect the affinity of the enzyme to the substrate (estrone). Nevertheless, it is necessary to study enzyme kinetic reactions with untreated microsomes to avoid possible alterations caused by detergents. Such studies with endogenous substrates have, to our knowledge, received very sparse attention. The kidney of the pig was chosen as the source of the enzyme because the post nuclear fraction of this tissue possessed specific activity higher than the liver with estrone as the substrate [4]. The present paper reports the results of the investigations on the mechanism of the forward reaction, namely the formation of estrone glucuronide, and the reverse reaction, which involves formation of estrone from its glucuronide and UDP^+. Both reactions take place by a sequential mechanism. Substrate analogs and nonsteroidal compounds inhibit estrone glucuronyltransferase noncompetitively, thereby indicating multiplicity of pig kidney microsomal glucuronyltransferases.

Materials and Methods

Chemicals

[4-^{14}C]Estrone (58 mCi/mmol) was obtained from the Radiochemical Center, Amersham, Bucks, England, uridine-5′-diphosphoglucuronate (UDPGlcUA) and uridine-5′-diphosphate (UDP) from Boehringer, Mannheim, Germany. Estrone glucuronide was generously supplied by Dr. Röhle, Bonn [5]. The purity of [4-^{14}C]estrone was checked in two different chromatographic systems.

Preparation of microsomes

The kidneys from freshly slaughtered female pigs were obtained from the local slaughterhouse. After removal of the capsule and connective tissue, the kidneys were cut into small pieces with a pair of scissors and homogenized with an Ultra Turrax (Janke & Kunkel, Staufen, Breisgau) in 0.25 M sucrose in 0.1 M Tris · HCl, pH 7.4. The homogenate (10%) was centrifuged at 600 × g for 10 min, at 8000 × g for 10 min and at 25 000 × g for 10 min, transferring the supernatants each time to new centrifuge tubes. The 25 000 × g supernatant was centrifuged at 150 000 × g for 30 min. The microsomal pellet was suspended in the homogenization medium and centrifuged to obtain the microsomal fraction; portions were kept at −20°C until further use. Protein was determined according to the method of Lowry et al. [6].

Biosynthesis of [4-^{14}C]estrone glucuronide

Radioactive estrone glucuronide for studies of the reverse reaction was synthesized by incubating 1 μCi [4-^{14}C]estrone with 2 mg UDPGlcUA and 4 ml 0.1 M Tris · HCl buffer, pH 8.0, containing 400 μg microsomal protein for 30 min at 37°C. After incubation, unreacted [4-^{14}C]estrone was quantitatively extracted with 20 ml water-saturated dichloromethane. The aqueous phase, containing estrone glucuronide, was evaporated under vacuum and the residue was taken up in 5 ml ethanol, shaken for 1 h and centrifuged to remove insoluble material. Purification and identification of the product was carried out as described by Rao et al. [4].

Determination of estrone glucuronyltransferase activity

The method for the determination of enzyme activity for the forward reaction was carried out according to the method described by Rao et al. [3]. [4-^{14}C]Estrone (about 70 000 cpm) and the appropriate amounts of nonradioactive estrone in ethanolic solution were pipetted into incubation tubes. One drop of propylene glycol was added, the ethanol was evaporated with a gentle stream of N_2 at 45°C. The appropriate amount of freshly prepared UDPGlcUA solution in 0.1 M Tris · HCl buffer, pH 8.0, was added to the tubes containing estrone and the total volume was brought to 0.8 ml with 0.1 M Tris · HCl buffer, pH 8.0. The incubation tubes were equilibrated for 15 min at 37°C. The reaction was started by pipetting 0.2 ml of microsomal suspension, equivalent to 100 μg of protein, in intervals of 5 s, and incubated for 5 min at 37°C.

After incubation the tubes were removed and kept in an ice-water bath. The tubes were then placed in a boiling water bath for 10 min and later transferred to an ice-water bath. The unreacted estrone was quantitatively (99.7%) removed by extracting the sample once with 10 ml of water saturated dichloromethane; 0.5 ml of the aqueous phase was pipetted into counting vials, 12 ml of Bray's scintillation fluid was added and the samples were counted in a Packard scintillation spectrometer with 10 000 counts preset in order to keep counting error below 1%. The efficiency for ^{14}C was 70%. Blank values, i.e. controls at zero time, were obtained as follows: Incubation tubes were prepared in the same way as for the test incubations, but were not incubated at 37°C; further processing was carried out as described for the test incubations. The variation coefficient for the assay was 6%. 1 unit of enzyme activity was defined as that amount which conjugated 1 μmol estrone per min; specific activity was defined as μmol estrone glucuronide formed per mg protein per min. The rate of glucuronide formation, v, is expressed as nmol product formed per 100 μg of protein per min, unless otherwise stated.

The formation of UDPGlcUA and estrone from estrone glucuronide and UDP (reverse reaction) was measured by incubating various amounts of nonlabeled estrone glucuronide and constant amount of radioactivity of [4-^{14}C]estrone glucuronide (about 50 000 cpm) with constant amounts of UDP in the presence of 500 μg microsomal protein for 30 min at 37°C in 0.1 M Tris · HCl buffer, pH 8.0. The total volume was 1 ml. The incubation was terminated by keeping the tubes in a boiling water bath for 10 min. The estrone formed was extracted with 2 ml of water-saturated ethyl acetate, while estrone glucuronide was not extracted to any measurable extent. A portion (1 ml) of ethyl acetate was mixed with 12 ml of Bray's scintillation fluid and counted in a Packard scintillation spectrometer with a preset of 10 000 counts. A series of estrone glucuronide concentrations was incubated with the microsomal fraction in the absence of UDP in order to measure the amount of estrone formed due to β-glucuronidase or other metabolizing enzymes. Estrone was not liberated during incubation with 500 μg microsomal protein in the absence of UDP, nor could any other metabolite of estrone be found. The extractability of estrone was checked by dissolving different amounts of [4-^{14}C]estrone in the incubation buffer as described under "forward reaction" and shaking with 2 ml ethyl acetate saturated with water; 95% estrone was extractible and the results were corrected to 100%.

Results

Properties of the enzyme

By the differential centrifugation of the kidney homogenate as described under Materials and Methods, the microsomes obtained, were found to contain no β-glucuronidase, pyrophosphatase or hydroxylating activity under the incubation conditions used. Microsomes prepared in this way possessed a specific activity of 0.03. The microsomal enzyme when kept at −20°C was active for more than 4 months; it was observed that the affinity of the enzyme to estrone increased slightly during the storage period as was inferred from measurements of the K_m values.

From preliminary experiments an apparent K_m value of 25 μM was calculated for UDPGlcUA at a constant concentration of 90 μM estrone and an apparent K_m of 3.5 μM for estrone at 800 μM UDPGlcUA. Subsequent experiments were then carried out at 90 μM estrone (at 26 times its K_m value) and at 800 μM UDPGlcUA (32 times its K_m value). The normalized substrate concentration (S') is the ratio of substrate concentration to K_m ($S' = S/K_m$). The Henri-Michaelis-Menten equation can then be expressed in terms of S' as

$$\frac{v}{V} = \frac{S/K_m}{1 + S/K_m} = \frac{S'}{1 + S'} = v'$$

thus the fraction of the V (v') reached at a given substrate concentration can be calculated [7]. For 800 μM UDPGlcUA, v' becomes 97% of V, and for 90 μM estrone 96% of V. Using these concentrations of the two substrates, the V obtained lies within the limits of the experimental error of the method [3].

Experiments carried out at 90 μM estrone and 800 μM UDPGlcUA with increasing amounts of microsomal protein and at different length of time exhibited zero-order kinetics up to 500 μg protein and up to 60 min incubation at 37°C (figure not shown).

Effect of pH

Assay of microsomal glucuronyltransferase in different buffers (0.1 M Tris/maleate, 0.1 M glycine/NaOH and 0.1 M Tris · HCl) revealed maximal activity in 0.1 M Tris · HCl buffer; a plateau of activity was observed between pH 7.3 and 8.7. Plotting the data on a semi-log scale gave pK values in the range of 6—7 and 9.5—10.5.

Influence of temperature on estrone glucuronide formation

Incubation of the enzyme with estrone and UDPGlcUA at varying temperatures showed maximal activity at 55°C. An Arrhenius plot of V against $1/T$ (Fig. 1) showed linearity of product formation between 2 and 45°C; the activation energy was calculated [8] to be 15.2 kcal/mol. Incubation of the microsomal protein for 15 min in the absence of both substrates resulted in a decrease of activity above 30°C; heating for 15 min at 48°C led to 50% loss of enzymic activity and complete inactivation at 70°C. Thus the two substrates (estrone and UDPGlcUA) have a stabilizing effect on the enzyme.

Influence of cations, EDTA and ATP on the activity of estrone glucuronyltransferase

Among all metal ions tested, 10 mM Mg^{2+} and 10 mM Ca^{2+} stimulated enzymic activity 30%. Cd^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} at a concentration of 10 mM inhibited the enzyme completely. 10 mM Fe^{2+} inhibited the enzyme 56% whereas 10 mM Fe^{3+} did not have any influence on enzymic activity. EDTA (0.01—1.0 mM) had no effect on the enzyme; ATP (1 mM) inhibited the enzyme 17%.

To get some insight into the action of Mg^{2+} on enzyme activity, studies were carried out at a constant saturating concentration of estrone (90 μM) and varying concentrations of UDPGlcUA in the presence and absence of 10 mM Mg^{2+}. The reaction increased 30% at high concentrations of UDPGlcUA (>1.6 mM); at low concentrations (<200 μM UDPGlcUA) however, a competitive type of inhibition was observed. Experiments with increasing concentrations of estrone and a constant concentration of UDPGlcUA (800 μM) showed 20—30% stimulation over the entire concentration range of estrone, compared to the control experiments (absence of Mg^{2+}), simultaneously carried out. From these results one may conclude that Mg^{2+} influences the UDPGlcUA binding site of the enzyme molecule as a nonessential agent. Hence further experiments were carried out in the absence of Mg^{2+}.

Influence of sulfhydryl reagents

Dithioerythritol and *N*-ethylmaleimide, from 0.01 to 1.0 mM, do not influence enzymic activity; 0.1 mM *p*-chloromercuribenzoate inhibits the enzyme 67%, 1 mM *p*-chloromercuribenzoate inhibits 92% and at 10 mM *p*-chloromercuribenzoate, complete inhibition was observed.

Studies on the mechanism of the forward reaction

As a prerequisite for the study of the mechanism of enzymatic reaction, experiments should be carried out with a purified enzyme. However, the difficul-

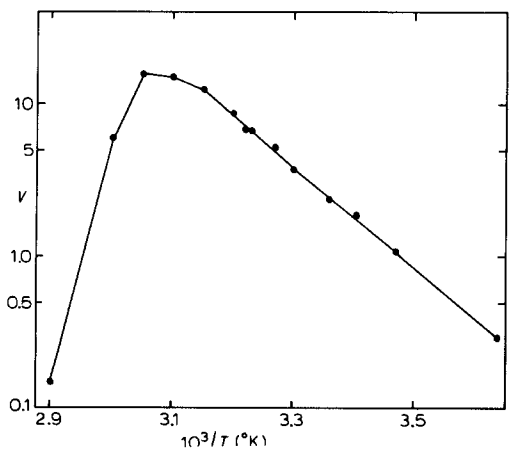

Fig. 1. Arrhenius plot of the velocity of estrone glucuronide formation. 90 μM estrone and 800 μM UDPGlcUA were incubated with 100 μg protein in 0.1 M Tris · HCl buffer, pH 8.0. Other details are mentioned under Materials and Methods. *V* is expressed as nmol glucuronide formed per 100 μg protein per min.

ties in obtaining a pure form of the enzyme from the microsomes are well known. In such cases adequate information can still be obtained provided the activators and inhibitors present in the enzyme preparation, the microsomes, do not affect the reaction under investigation and other enzymatic reactions involving the substrates, are minimal. The nomenclature used in the present paper is taken from Cleland [9,10]. The reciprocals of the observed velocities were plotted against the reciprocals of the substrate concentrations in the presence and absence of different compounds. The regression lines, as suggested by Segel [7], were calculated with a program of the method of least squares with an Olivetti desk computer. The intersections on the y-axes, x-axes and the slopes were calculated by the program. Each experimental point in the diagrams is the uncorrected mean of the measured duplicate. Experiments were repeated to obtain consistent results.

Using increasing concentrations of estrone with several fixed concentrations of UDPGlcUA a family of reciprocal plots was obtained which intersect at a point above the x-axis and pivots clockwise with increasing concentrations of UDPGlcUA. The double reciprocal plots were found to be linear in all cases (Fig. 2). Replotting the reciprocal of the concentrations of UDPGlcUA against the reciprocal of v at constant concentrations of estrone results in a family of lines which is symmetrical to that shown in Fig. 2. As can be seen from Fig. 2 increasing concentrations of the fixed substrate increase the apparent V for the varied substrate, and the affinity of the enzyme to the varied substrate also increases. Replotting the slopes of 1/[estrone] against 1/[UDPGlcUA] yields a K_{UDPGlcUA} of 254 μM. Similarly the slope of 1/[UDPGlcA] against 1/[estrone] gives a K_{estrone} of 6.6 μM.

The V value was obtained by replotting the apparent V values versus the concentrations of the nonvaried substrate. Solving for V, a value of 328 pmol/(100 μg protein · min) is obtained. From these replots also $\alpha K_{\text{estrone}}$ and $\alpha K_{\text{UDPGlcUA}}$ can be obtained, which were 1.4 μM and 35.2 μM, respectively. The values of α calculated from αK and K values [7] for estrone UDPGlcUA were 0.21 and 0.14, respectively. In both cases the factor α is less than unity, i.e., the binding of one substrate increases the affinity of the enzyme for the second substrate. Since the ratio of apparent K_m values to apparent V values is not constant, the mechanism is sequential, which means that both substrates must bind to the enzyme before any product is released. Plotting the data according to Hill [11], $\log S$ against $\log \frac{v}{V-v}$, give slopes of $n \approx 1$, i.e. not more than one molecule of either substrate binds to the enzyme under the incubation conditions described.

Product inhibition studies

Since product formation does not distinguish between different sequential mechanisms it was felt that product inhibition studies would give some information about the mechanism of the glucuronidation reaction. Under initial velocity conditions the products of the forward reaction, UDP and estrone glucuronide, were used as inhibitors. These studies were somewhat hampered by the fact that estrone glucuronide is not commercially available and could be synthesized only in limiting amounts; therefore the experiments were carried out at saturation concentration of the nonvaried ligands, namely estrone and

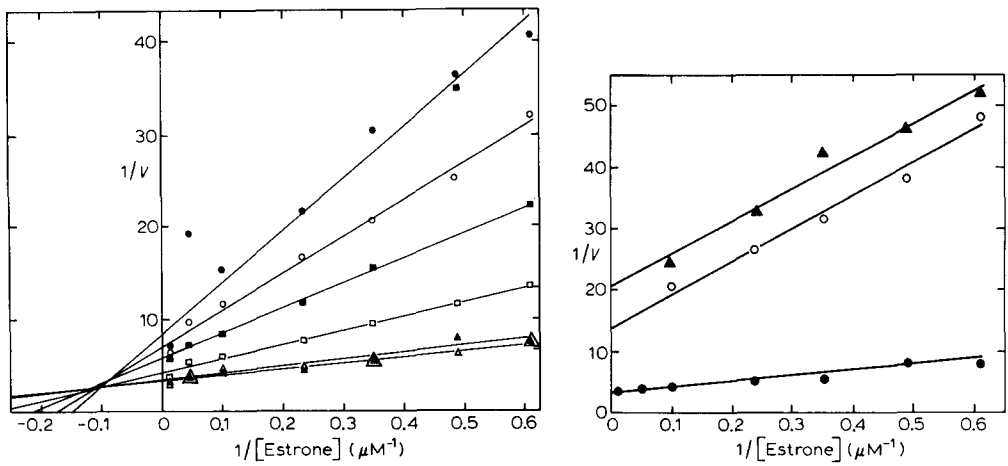

Fig. 2. Initial velocity pattern with estrone as the varied substrate. UDPGlcUA: ●———●, 20 μM; ○———○, 27 μM; ■———■, 40 μM; □———□, 80 μM; ▲———▲, 320 μM; △———△, 800 μM. Initial velocity, v, is expressed in this and other figures, unless otherwise stated, as nmol estrone glucuronide formed per 100 μg protein per min. Protein concentration, 100 μg/ml. Other details are mentioned under Materials and Methods.

Fig. 3. Reciprocal plot with estrone as the varied substrate and UDP as inhibitor. The UDPGlcUA concentration was 800 μM, protein concentration 100 μg/ml. ●———●, without UDP; ○———○, 200 μM UDP; ▲———▲, 300 μM UDP. Other details are mentioned under Materials and Methods.

UDPGlcUA, respectively. Fig. 3 shows the product inhibition pattern with UDP as the inhibitor and UDPGlcUA as the constant substrate at saturating concentration; a noncompetitive inhibition was observed. Using estrone at saturating concentration (90 μM) and varying concentrations of UDPGlcUA the inhibition with 50 μM UDP was linear and uncompetitive. With estrone as the variable substrate and with saturating concentrations of UDPGlcUA (800 μM), 200 μM estrone glucuronide did not inhibit the reaction, whereas with UDPGlcUA as the variable and estrone as the constant substrate (90 μM) the results were suggestive of a competitive type of inhibition (Figures not shown).

Reverse reaction

Incubation of increasing concentrations of estrone glucuronide in the presence of constant concentrations of UDP, both products of the forward reaction, yield an initial velocity pattern which is also sequential (Fig. 4). Replotting the resulting slopes as secondary plots gives a $K_{\text{estrone glucuronide}}$ of 15.3 μM and a K_{UDP} of 1.73 mM. Replotting the apparent $1/V$ versus 1/[UDP] gives a V of 1.3 pmol/(0.1 mg protein · min) (figures not shown).

Effect of steroids and nonsteroid compounds on the glucuronidation of estrone

To test the influence of other endogeneous substrates of glucuronyltransferases as well as xenobiotics, constant concentrations of these compounds were incubated with the enzyme in the presence of saturating, constant concentration of UDPGlcUA (800 μM) and varied concentrations of estrone; the results of these incubations with 10, 15 and 20 μM estradiol-17β are presented in Fig. 5

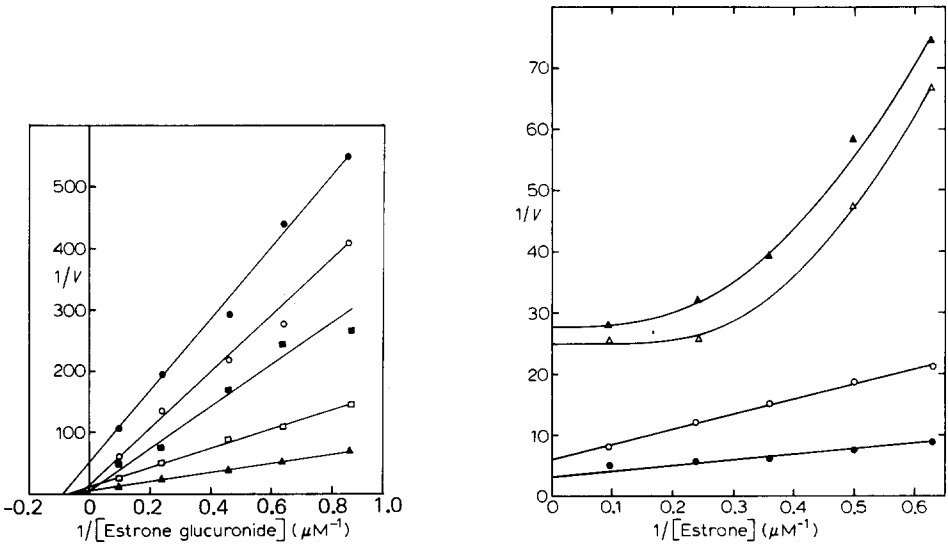

Fig. 4. Initial velocity pattern with estrone glucuronide as the varied substrate. Initial velocities are expressed as nmol of [4-^{14}C]estrone formed per min, per 100 μg microsomal protein, protein concentration 500 μg/ml. UDP: ●———●, 50 μM; ○———○, 70 μM; ■———■, 100 μM; □———□, 200 μM; ▲———▲, 500 μM. Other details are mentioned under Materials and Methods.

Fig. 5. Reciprocal plot with estrone as the varied substrate and fixed concentration of estradiol-17β as inhibitor, UDPGlcUA concentration was 800 μM, protein concentration 100 μg/ml. Estradiol-17β: ●———●, none; ○———○, 10 μM; △———△, 15 μM; ▲———▲, 20 μM. Other details are mentioned under Materials and Methods.

and with bilirubin, a nonsteroid, at 50 and 200 μM in Fig. 6. The effect of other aglycons incubated under the same incubation conditions are summarized in Table I. All compounds tested produced linear noncompetitive inhibition to

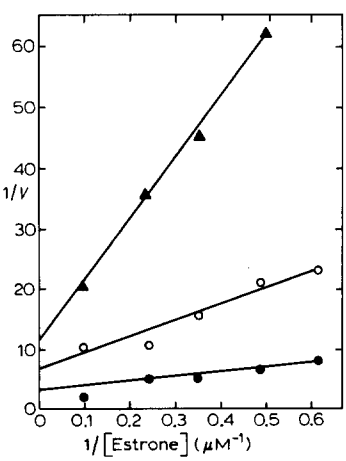

Fig. 6. Reciprocal plot with estrone as the varied substrate and fixed concentrations of bilirubin as inhibitor. The UDPGlcUA concentration was 800 μM, protein concentration 100 μg/ml. ●———●, without bilirubin; ○———○, 50 μM bilirubin; ▲———▲, 200 μM bilirubin.

TABLE I

INFLUENCE OF STEROIDS AND NONSTEROID COMPOUNDS ON THE GLUCURONIDATION OF ESTRONE

Compound	Concn. (μM)	Type of inhibition	K_i (μM)
Estradiol-17β	5	Noncompetitive, linear	15.0
	10	Noncompetitive, linear	10.9
	15	Noncompetitive, nonlinear	5.0
	20	Noncompetitive, nonlinear	6.2
Estradiol-17α	25	Noncompetitive, linear	55.2
	50	Noncompetitive, linear	13.8
Estriol	20	Noncompetitive, linear	110
	40	Noncompetitive, linear	53.3
Testosterone	25	Noncompetitive, linear	47.0
	50	Noncompetitive, linear	291
Phenolphthalein	50	Noncompetitive, linear	8.6
Bilirubin	50	Noncompetitive, linear	50.0
	200	Noncompetitive, linear	83.4
o-Aminophenol	50	No inhibition	
	200	No inhibition	
p-Nitrophenol	50	No inhibition	
	100	No inhibition	

varying degrees except *o*-aminophenol and *p*-nitrophenol which did not have any influence on the conjugation of estrone. Higher concentrations of estradiol-17β produced nonlinear noncompetitive inhibition. The K_i values of estradiol-17β and of phenolphthalein are in the range of the K_m for estrone. With estradiol-17α, estriol, testosterone and bilirubin as inhibitors, the K_i values varied with different concentrations and were in the range of 50 to 290 μM.

Discussion

The estrone glucuronyltransferase from pig kidney microsomes can be stimulated by Mg^{2+} and Ca^{2+}; these cations function as nonessential activators, in that their presence is not essential for enzymic activity. Bivalent metals such as Fe^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} in concentrations of 10 mM completely inhibit the enzyme, probably by complexing with free SH groups of a cysteine residue of the enzyme; this is also in good agreement with the total inhibition caused by 10 mM *p*-chloromercuribenzoate, a sulfhydryl reagent. Since *N*-ethylmaleimide has no influence on the enzyme, one may speculate that the SH group is hidden in the enzyme and cannot readily be attacked by an alkylating agent. Further information in this respect is also gained from the pH-curve, from which pK values in the range of 6—7 and 9.5—10.5 are obtained. These pK values point to the involvement of imidazole or ϵ-amino groups and phenolic groups with the enzymic activity, but not to direct involvement of cysteine, which has a pK value of 8—8.5. The optimum pH of the enzyme between 7.3 and 8.4 agrees well with that observed for bilirubin glucuronyltransferase from cat liver microsomes [12] which lies between pH 8.0 and 8.8.

As has been mentioned under Results the microsomal preparation does not catalyze any other reaction under the incubation conditions employed which could in some way interfere with the glucuronidation reaction. If pyrophospha-

tases interfered with the glucuronidation reaction by degrading UDPGlcUA, linearity of glucuronide formation with respect to time and amount of protein could not have been obtained. Likewise ATP which is also a substrate for pyrophosphatase would lead to a stimulation of the glucuronyltransferase, since it would compete with UDPGlcUA for the binding site on the pyrophosphatase. Thus more UDPGlcUA would be available for the glucuronyltransferase resulting in an increase in the rate of glucuronidation of estrone. However, ATP in equimolar concentration of UDPGlcUA inhibits the glucuronidation of estrone by 17%.

Linearity of the Arrhenius plot between 2 and 45°C points to the fact that changes in the fluidity of the lipid portion of the endoplasmic reticulum membrane caused by increasing temperature do not influence the activity of estrone glucuronyltransferase.

According to Cleland [9] enzymic reactions can be divided into two main groups: (a) sequential mechanism, where all reactants combine with the enzyme before one product is released and (b) Ping-Pong mechanism where one or more products can be set free before all substrates combine with the enzyme. In the present investigation the lines of initial velocity intersect on the left of the ordinate above the abscissa which is indicative of a sequential mechanism. For the reverse reaction a similar type of reaction mechanism was also observed. The K_m values of the substrates for the reverse reaction are 2—7 times higher than those for the forward reaction; the obtained V value is 1/300 that of the forward reaction. Product inhibition studies were carried out to gain more information about the reaction mechanism. When the studies were carried out at saturating concentration of the nonvaried substrate (estrone or UDPGlcUA), the following pattern of inhibition in the forward direction was obtained:

Substrate	Inhibiting product	Type of inhibition
Estrone (E_1)	Estrone glucuronide (E_1G)	No inhibition
	UDP	Noncompetitive
UDPGlcUA	Estrone glucuronide	Competitive
	UDP	Uncompetitive

On comparison of these preliminary data with the nomenclature proposed by Cleland [10], it is possible that the enzyme follows the pattern of an iso-Theorell-Chance mechanism:

E_1 ↓	UDPGlcUA ↓	E_1G ↑	UDP ↑
(E)	(E-E_1)	(Complex)	(E-UDP)	(E)

In this mechanism practically no central (E-E_1-UDPGlcUA) (E-E_1G-UDP) complex exists, although the possibility of the isomerization of the enzyme cannot be excluded.

In order to evaluate the kinetic importance of a possible existence of a central complex in this system the ratio of central complex to E-estrone was calculated as proposed by Janson and Cleland [13], assuming an ordered mechanism where estrone would combine with the enzyme first:

$$R = \frac{\Sigma \text{ vertical coordinates of the crossover points}}{\Sigma\ 1/V}$$

Thus one may independently arrive at a conclusion that the central complex is essentially zero, calculating the ratio R by dividing the sum of the vertical intersection coordinates of the original reciprocal plots of initial velocity of the forward and the reverse reaction, by the sum of the reciprocal V values in both directions. For a Theorell-Chance mechanism where the central complex is at practically zero level, one should arrive at a value of 0; for an ordered rapid equilibrium system the value should be 1. Taking the data from Figs. 2 and 4, R was calculated to be 0.018, a figure which is closer to 0 than 1, and which is a good indication of a Theorell-Chance mechanism.

The R value is independent of an isomerization of a noncentral transitory complex and since the V value for the forward reaction is much higher than that for the reverse reaction, the proportion of the enzyme present as a central complex in the slower (reverse) direction, with both substrates at saturating concentration will be equal to R, in this case 0.018.

From these considerations one may conclude that for the forward reaction as well as for the reverse reaction increasing concentrations of one substrate will increase the affinity of the other substrate to the enzyme, consequently increasing the V value of the reaction. Since the K_m values of estrone glucuronide and UDP are much higher than those for estrone and UDPGlcUA, and since the V value of the reverse reaction is only 1/300 that of the forward reaction, one may assume that the formation of estrone from estrone glucuronide plays a minor role. However, in conjunction with β-glucuronidase which is present in the kidney cell of the pig [3] hydrolysis of estrone glucuronide could take place. These results point to the fact that estrone glucuronyltransferase catalyzes the forward reaction, i.e. the formation of estrone glucuronide; although the reverse reaction is detectable, the rate is insignificant.

Much speculation has been made about the multiplicity of UDP-glucuronyltransferases. Recent literature however, does indicate that there is a general agreement about the existence of multiple UDP-glucuronyltransferases. The inhibition studies with different steroidal and nonsteroidal compounds support the concept of multiplicity, since none of the acceptors tested, inhibited the formation of estrone glucuronide competitively. In the present investigation the two estrogens (estrone and estradiol-17β) seem to be conjugated by two different enzymes; however it is doubtful, that the enzyme has such narrow specificity although it cannot be altogether excluded.

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