SUMMARY

1. A method for the subcellular fractionation of intestinal mucosa is described. The subcellular fractions were characterized by electron microscopy and by determining the distributions of cytochrome oxidase and alkaline phosphatase in the subcellular fractions.

2. The subcellular distribution of peptidases hydrolysing L-leucylglycine and glycylglycine has been determined in intestinal mucosa. Most of the peptidase activity was located in the supernatant fraction. The remaining activity was found mainly in the microsomal fraction, with some in the mitochondrial fraction.

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Biochem. J. (1963) **88**, 168

Enzymic Formation of Oestrone 3-Glucuronide by Rabbit-Liver Microsomes

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It has been known for many years that phenolic steroids are excreted in a conjugated form in urine. An oestriol conjugate was first isolated from human pregnancy urine by Cohen & Marrian

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(1936), and it was found that one glucuronic acid residue was attached to each steroid molecule in ring D (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1950). More recently, Carpenter & Kellie (1962) have shown, by comparison with authentic derivatives, that pregnancy urine contains oestriol

16 α -monoglucuronide as well as oestriol 17 β -monoglucuronide; Neeman & Hashimoto (1961, 1962) also isolated an oestriol monoglucuronide, which they demonstrated to be the oestriol 16 α -monoglucuronide. Felger & Katzman (1961) reported the identification of a diglucuronide of oestriol from the same source, and Beling (1962) obtained evidence of the presence of oestriol 3-monoglucuronide. Oestriol glucuronides have also been identified in human meconium (Menini & Diczfalussy, 1960), in human cord blood, amniotic fluid and in urine of the newborn (Troen, Nilsson, Wiquist & Diczfalussy, 1961). Although there is evidence of the existence of oestradiol and oestrone glucuronides in pregnancy urine, none of these has yet been isolated.

The extensive studies by Dutton & Storey (for references see Dutton & Storey, 1962) have revealed that enzymic glucuronidation of many hydroxy compounds occurs in liver. This reaction is catalysed by the glucuronyltransferase and requires the presence of uridine diphosphate glucuronic acid from which the glucosiduronyl residue is transferred to an aglycone. The glucuronyltransferase has been found in the microsomal fraction of liver tissue (Dutton & Storey, 1954; Isselbacher, 1956). Isselbacher (1956) has studied the formation of glucuronides of neutral steroids *in vitro* and indicated that phenolic steroids may also be conjugated by microsomal systems.

In the experiments to be described here the formation of oestrone 3-glucuronide was investigated in detail, by using rabbit-liver microsomes. Oestrone, which has only one hydroxyl group, was chosen as the aglycone to avoid ambiguity about the reaction product and thus to facilitate kinetic studies.

MATERIALS AND METHODS

Solvents. All solvents were redistilled before use. Propylene glycol was distilled *in vacuo*.

Uridine diphosphate glucuronic acid. The '95% grade' preparation was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A.

Oestrone. This was obtained from Schering A.G., Berlin West, and was found to be chromatographically pure.

Preparation of oestrone 3-monoglucuronide. Oestrone 3-monoglucuronide triacetyl methyl ester was prepared according to Wotiz, Smakula, Lichtin & Leftin (1959), using the methods of Schapiro (1939) and of Meystre & Miescher (1944). The triacetyl methyl ester was deacetylated as described by Diczfalussy, Franksson, Lisboa & Martinsen (1962).

Preparation of microsomes. A rabbit (2.0 kg.) was killed by a blow on the neck and the liver (65 g.) removed immediately. The tissue was cut into small pieces and homogenized in five portions, each with 52 ml. of ice-cold 0.25 M-sucrose, in a Potter-Elvehjem all-glass homogenizer. All subsequent operations were carried out at approx. 4°.

The homogenate was centrifuged at 1000g for 10 min. and the supernatant at 10 000g for 20 min. The cloudy supernatant was next centrifuged at 100 000g for 60 min. and the precipitate suspended in 240 ml. of 0.25 M-sucrose. After centrifuging at 100 000g for 60 min. the microsomal fraction was resuspended in 65 ml. of 0.154 M-KCl; 1 ml. of this suspension therefore corresponded with approx. 1 g. of fresh liver tissue. Several preparations were made in this manner and appeared to retain their β -glucuronosyltransferase activity for at least 4 days when stored at 2-4°.

Incubation technique. Oestrone dissolved in propylene glycol (0.2 ml.) was pipetted into a 15 ml. tube to which were added phosphate buffer (0.15 M; 1.7 ml.; pH 8.2), UDP-glucuronic acid in water (0.1 ml.) and the microsome suspension. If necessary, the total volume was made up with KCl (0.154 M) to 3.0 ml. The tubes were shaken in a water bath at 37°.

Methods of extraction and estimation of oestrone 3-glucuronide. Method A. The incubation mixture (3.0 ml.) was extracted with ether (3 $\times$ 8 ml.) to remove the unconjugated oestrone and then treated with ethanol (12 ml.). The precipitated protein was removed by centrifuging at 500g for 10 min. and the ethanolic solution evaporated *in vacuo* to dryness. The residue was taken up in ice-cold HCl (2 N; 4 ml.) and the solution extracted with ethyl acetate (3 $\times$ 4 ml.) in the cold. The recovery of oestrone 3-glucuronide by this extraction was found to be 90%. The combined ethyl acetate extracts were washed with HCl (2 N; 4 ml.) at 4° and evaporated *in vacuo* to dryness. The residue, which contained the conjugated oestrone, was hydrolysed with HCl (1.6 N; 4 ml.) for 60 min. at 100°. The cooled aqueous solution was extracted with benzene (2 $\times$ 5 ml.) and the benzene then extracted with NaOH (2 N; 2 $\times$ 5 ml.). Sodium hydrogen carbonate (1 M; 40 ml.) was added to the alkaline solution and the oestrone extracted with ether (2 $\times$ 40 ml.). The ether was dried over Na₂SO₄ and evaporated *in vacuo* to dryness. Oestrone was determined by the Kober reaction (Brown, 1952; Bauld, 1954), as modified by Nocke (1961). Incubation mixtures in which oestrone only was omitted give blank values equivalent to approx. 0.1 μ g. of oestrone. From recovery experiments, the overall yield was calculated to be approx. 80%.

Method B. The incubation mixture was extracted with ether and the protein precipitated with ethanol. The residue after evaporation of the ethanolic solution was then hydrolysed with HCl and extracted with benzene as in method A, but the extraction with ethylacetate was omitted. The benzene extract was next washed with water (2 $\times$ 5 ml.) to remove propylene glycol and then extracted with NaOH. The alkaline solution was treated as in method A.

Paper chromatography. Chromatograms (Schleicher and Schüll paper no. 2043 b Mgl) were run for 4-5 hr. in the system 2-methylpropan-2-ol-ethylene dichloride-acetic acid-water (5:15:6:14, by vol.) after equilibration for 24 hr. Oestrone 3-monoglucuronide was located on the chromatograms by the Zimmermann reaction in the following way. The paper was dipped into ethanolic 2.4 N-KOH and blotted, then dipped into ethanolic 2% *m*-dinitrobenzene and again blotted; the paper was then dried in warm air. The compound appeared as a violet spot.

Spectrophotometric measurements. Extinction was measured in a 10 mm. cuvette with a Beckman spectrophotometer model DU.

RESULTS

Formation of oestrone 3-glucuronide by a preparation of rabbit-liver microsomes. Oestrone ($0.61 \mu\text{mole}$) was incubated for 1 hr. in phosphate buffer with UDP-glucuronic acid ($0.63 \mu\text{mole}$) and 1.0 ml. of the microsome suspension. To obtain enough material, the residues from the ethanolic extract from three experiments were combined and taken up in ice-cold HCl ($2N$; 5 ml.). This was then extracted with ethyl acetate ($3 \times 5 \text{ ml.}$), whilst the solution was kept near 0° . The combined ethyl acetate extracts were washed with HCl ($2N$; 5 ml.) and evaporated *in vacuo* to dryness. One-tenth of this residue was hydrolysed before carrying out the Kober reaction, which showed a total of $77 \mu\text{g.}$ of oestrone, corresponding with $127 \mu\text{g.}$ of oestrone 3-glucuronide. For further identification, approximately one-fifth of the residue was chromatographed on paper in the system 2-methylpropan-2-ol-ethylene dichloride-acetic acid-water. The substance was located by using the Zimmermann reaction and showed the same R_F (0.53) as authentic oestrone 3-glucuronide; it did not give the blue colour shown by phenols with Folin-Ciocalteu reagent. Four-fifths of the residue was taken for infrared spectroscopy; the spectrum obtained was identical with that of authentic oestrone 3-glucuronide.

Kinetic studies of the formation of oestrone 3-glucuronide. After it had been demonstrated that oestrone 3-glucuronide is, in fact, formed when oestrone is incubated with UDP-glucuronic acid and rabbit-liver microsomes, it was decided to study the kinetics of this reaction. Since quantitative estimations of oestrone 3-glucuronide would be rather laborious, a measure of the oestrone conjugated was obtained from determination of oestrone liberated by acid hydrolysis.

Before kinetic studies could be started it was essential to find out if the oestrone 3-glucuronide

formed is hydrolysed by microsomal preparations. For this purpose, oestrone 3-glucuronide ($50 \mu\text{g.}$) was incubated in the usual way with microsomes and the mixture extracted with ether-chloroform. No more than $0.2 \mu\text{g.}$ of oestrone was found in this extract, thereby indicating that practically no hydrolysis of oestrone 3-glucuronide occurs under

Fig. 1. Effect of pH on the formation of oestrone 3-glucuronide. Oestrone ($27 \mu\text{g.}$, $0.1 \mu\text{mole}$) was incubated for 1 hr. with $0.4 \mu\text{mole}$ of UDP-glucuronic acid and 1.0 ml. of microsomes in 1.7 ml. of phosphate buffers ($0.15M$) at various pH values. Estimation of oestrone 3-glucuronide followed method B.

Fig. 2. Effect of time of incubation on the formation of oestrone 3-glucuronide. Oestrone ($27 \mu\text{g.}$, $0.1 \mu\text{mole}$) was incubated in 1.7 ml. of phosphate buffer ($0.15M$; pH 8.2) with $0.4 \mu\text{mole}$ of UDP-glucuronic acid and 1.0 ml. of microsomes for various times. Estimation of oestrone 3-glucuronide followed method A.

Table 1. Effect of the concentration of propylene glycol on the formation of oestrone 3-glucuronide

Oestrone ($27 \mu\text{g.}$), dissolved in various amounts of propylene glycol, was incubated for 1 hr. in 1.7 ml. of phosphate buffer ($0.15M$; pH 8.2) with $0.3 \mu\text{mole}$ of UDP-glucuronic acid and 1.0 ml. of microsomes.

Propylene glycol added (ml.)	Amount of oestrone conjugated* (μg.)
0.05	6.6, 6.6
0.10	5.9, 6.2
0.20	6.0, 5.8
0.30	6.2, 5.5

* Determined after acid hydrolysis of the oestrone 3-glucuronide formed (method B).

Fig. 3. Lineweaver-Burk plot for the formation of oestrone 3-glucuronide. Various amounts of oestrone in 0.2 ml. of propylene glycol were incubated for 1 hr. in 1.7 ml. of phosphate buffer (0.15M; pH 8.2) with 0.345 μ -mole of UDP-glucuronic acid and 1.0 ml. of microsomes. Estimation of oestrone 3-glucuronide followed method A.

the conditions of the experiment. It was found in a similar experiment that oestradiol 3,17 β -diglucuronide also was not hydrolysed.

Cooke & Taylor (1961) reported that the concentration of propylene glycol influenced the degree of conjugation of progesterone by microsomal preparations. Since propylene glycol was used in the present study it appeared necessary to examine its effect on the yield of oestrone 3-glucuronide. The results (Table 1) show that the conjugation of oestrone was only slightly affected.

The effect of pH on the formation of oestrone 3-glucuronide was studied in the range 6.8–9.2. Fig. 1 shows that there is maximum of conjugation between pH 8.0 and 8.2. That conjugation of oestrone by the microsomal preparation is a function of time is shown in Fig. 2. Under the conditions described, very little oestrone was conjugated by further incubation after 1 hr. The effect of substrate concentration on the formation of oestrone 3-glucuronide was studied in the concentration range 10.1–40.6 μ M. The reaction velocity followed Michaelis-Menten kinetics. The Michaelis constant was determined by the method of Lineweaver & Burk (1934) (Fig. 3). It was found to be 9.7×10^{-5} M.

DISCUSSION

The aim of this work was to find if the liver-microsomal system is effective in the formation of glucuronides of phenolic steroids as it is with other phenolic substances. Oestrone 3-glucuronide was identified as the product of the reaction with oestrone, thus demonstrating that oestrone is in fact conjugated by the microsomal enzyme. The affinity between the substrate and the glucuronyl-transferase is comparatively strong, as evidenced by the low K_m , found to be 97 μ M for oestrone. The pH optimum of the reaction was at 8.0–8.2, which does not differ significantly from that found by Dutton & Storey (1954), who used mouse-liver microsomes with *o*-aminophenol as substrate. However, Isselbacher (1956), working with a five-fold-purified enzyme preparation from guinea-pig liver, observed a pH optimum of 7.6 for the conjugation of tetrahydrocortisone. Further work is necessary to decide whether the difference in pH optimum is a reflexion of more than one transferase being involved in the conjugation of neutral and phenolic steroids or is merely due to different experimental conditions.

Evidence has been accumulating that there are oestrogen glucuronides in pregnancy urine that have the glucuronic acid residue attached to the phenolic hydroxy group (Beling, 1962) or to an alcoholic hydroxy group in the ring D (Carpenter & Kellie, 1961, 1962). It appears therefore of interest to find whether the phenolic and alcoholic hydroxy groups in the oestrogen molecule are conjugated at different rates. Preliminary experiments indicated that under similar experimental conditions oestradiol-17 β 3-methyl ether is conjugated to a less degree than is oestrone. This result suggests that in rabbit-liver microsomes the rate of glucuronidation may be higher for the phenolic hydroxy group than for the 17-hydroxy group.

The formation of oestrogen glucuronides is not confined to liver tissue; it has also been found to occur in the intestine (Lehtinen, Nurmikko & Hartiala, 1958; Lehtinen, Hartiala & Nurmikko, 1958; Diezfalusy, Franksson & Martinsen, 1961; Schürholz & Staib, 1961; Diezfalusy *et al.* 1962). In particular, Diezfalusy *et al.* (1961) injected 50 mg. of oestriol into an isolated jejunal loop with the arterial blood supply intact and found a concentration in the effluent venous blood of 7 μ g. of conjugated oestriol/ml., which was identified as oestriol 16- or 17-glucuronide; they also detected large quantities of conjugated oestriol in the intestinal wall. Diezfalusy *et al.* (1962) in a similar experiment injected 20 mg. of oestradiol-17 β and identified oestrone 3-glucuronide in extracts of the intestinal wall and of the effluent venous blood. Glucuronyl-transferase activity was found in kidney tissue

comparable with that of liver tissue in guinea pigs (Dutton, 1959). To account for the large quantities of conjugated oestrogens excreted in the urine during pregnancy it will be necessary to know the rates of glucuronidation of these compounds by various organs.

SUMMARY

1. Oestrone 3-glucuronide has been identified as the reaction product after incubation of oestrone with a rabbit-liver microsomal preparation, uridine diphosphate glucuronic acid being used as the glucuronic acid donor. The formation of this compound was studied under various experimental conditions.

2. The reaction showed a maximum at pH 8.0–8.2, and K_m for oestrone was found to be $9.7 \times 10^{-5} M$.

3. The results are discussed in relation to the general problem of the physiological production of oestrogen glucuronides.

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Biochem. J. (1963) **88**, 172

The Binding of Pyridoxal 5-Phosphate to Aspartate Aminotransferase of Pig Heart

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In previous studies of the effects of sulphate and phosphate esters of oestrogens on the reconstitution of aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) prepared by the method of O'Kane & Gunsalus (1947), the enzyme was freed from the coenzyme by incubation

at 60° for 1 min. in the presence of 20 % (w/v) ammonium sulphate (Scardi, Magno & Scarano, 1960; Scardi, Iaccarino & Scarano, 1962). In later work the enzyme was prepared by the method of Jenkins, Yphantis & Sizer (1959) that permits the preparation in a relatively simple way of large