

Duodenal Glucuronide Synthesis

I. Identification of Estradiol Glucuronide as a Conjugation Product of Estradiol by the Rat Duodenal Mucosa. Qualitative Studies

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Upon incubating estradiol with slices of duodenum taken from 5 rats a product with the properties of estradiol glucuronide is formed. This product which is extractable from acid solution by ethylacetate gives after hydrolysis with β -glucuronidase chromatographically a qualitative reaction for estradiol and glucuronic acid.

Previous studies by one of us^{1,2} have shown that in addition to the liver also mucosal specimens taken from various regions of the gastrointestinal tract are able to conjugate glucuronides (*in vitro*) in the presence of *o*-aminophenol. According to these observations the intestinal glucuronide synthesis participates in the detoxication processes of the organism.

Feeding of another glucurogenic substance, cinchophen, to a dog leads to increased glucuronide contents of portal blood also under conditions when the bile flow into the intestine is prevented³. Recently we have also been able to identify phenolphthalein-glucuronide as a conjugation product of the duodenal mucosa⁴.

In the present work estradiol is used as the substrate. Its conjugation to a glucuronide by the duodenal mucosa *in vitro* has been demonstrated.

MATERIAL AND METHODS

Tissue specimens. Rats were used in these experiments. The animals were killed by a blow on the head. The tissue specimens were removed immediately, the size of specimens kept equal by means of a starfish. The samples were first placed into the ice cold incubation mixture.

Estradiol. Crystallized estradiol (Sigma Co., 4.9 mg) was dissolved in 2 ml of propylene glycol⁵.

Incubation. This was carried out in the Warburg apparatus at 37°C over a period of 90 min. The incubation solution (Krebs-Ringer) contained 100 mg glucose and 112 mg sodium lactate per 50 ml of the mixture. Into each Warburg flask were transferred 2.9 ml of this Ringer solution, 0.1 ml of the estradiol propylene glycol solution (0.9 μ mole estradiol) and after this the tissue specimens (approximate dry weight 8–10 mg). Gas phase was 5 % carbon dioxide in oxygen. As controls parallel runs were made with the incubation solution without tissue slices and also with tissue slices but without estradiol.

Sampling. After incubation the contents of 2 Warburg flasks were combined to make one sample for further concentration and chromatographic analysis. The tissue slices were removed from the incubation media and washed with 2 ml Ringer solution (6 ml + 2 ml).

Deproteinization. The incubation mixture was heated at 60°C for 3 min and centrifuged thereafter.

*Extraction of free estradiol*⁵. The clear protein-free supernatant was extracted with 10 ml of benzene (3 × 10 min). The precipitate left after the previous centrifugation was washed with absolute alcohol (1 ml), centrifuged and the alcohol supernatant added to the benzene solution. This was then evaporated in vacuum.

Extraction of estradiol glucuronide. The water phase left after benzene extraction was made 2 N with HCl and the glucuronide was extracted with ethylacetate (10 ml, 3 × 10 min). The ethylacetate was removed in vacuum.

Hydrolysis. To the ethylacetate residue were added acetate buffer (2 ml, 0.1 M, pH 4.5) and β -glucuronidase enzyme preparation (2 mg, Worthington Biochem. Corp.). The hydrolysis was carried out over a period of 24 h at 37°C.

Extraction of the estradiol liberated by hydrolysis. After the hydrolysis with β -glucuronidase the incubation solution was reextracted with benzene (7 ml, 3 × 10 min). Benzene was removed in vacuum as usual.

Glucuronic acid. The glucuronic acid made free by hydrolysis and after extraction with benzene is left in the water phase. This was evaporated to dryness and subjected for further analysis.

Paper chromatography. 1 ml of a solvent (ethylacetate/benzene) was added to each residue and the solutions were used for chromatographic analyses. These were carried out according to the descending chromatography method. A mixture of butanol, glacial acetic acid and water was used as an irrigant; 24 h, Paper Whatman No. 1. Estradiol spots were identified on the paper by a saturated aqueous solution of *p*-nitrobenzene-azodimethoxy-aniline (Fast Black Salt K, Heftman, 1950). Glucuronic acid was identified with the AgNO₃-ammonia reagent (1 vol. 0.1 N AgNO₃, 1 vol. 5.0 N NH₃-solution, 1 vol. 2 N NaOH).

RESULTS

From the acidified incubation mixture a compound was extracted with ethylacetate which, on hydrolysis with β -glucuronidase, gave spots for free estradiol and glucuronic acid.

Estradiol glucuronide. The nonhydrolyzed residue after evaporation of the ethylacetate extract failed to give a violet spot on the paper after fixation with Fast Black Salt K (Fig. 2. H, I, J,). This reagent is known to produce such colour with free estradiol⁶. This finding indicates that the estradiol is in a conjugated form in the ethylacetate extract. Spots F and G in Fig. 2 were obtained using 100 μ g and 150 μ g pure estradiol. From the nonhydrolyzed residue no dark brown spot characteristic for glucuronic acid was formed with the ammonia silver nitrate solution (Fig. 3. A, B, C). The small spots seen at A, B and C are probably caused by some carbohydrate impurities reacting with the potent silver nitrate reagent. The R_F -values of these are distinct from those given by pure glucuronic acid. Spot D corresponds to 150 μ g of pure glucuronic acid.

Estradiol and glucuronic acid liberated by hydrolysis. After the hydrolysis a compound dissolved in benzene which on the chromatogram appeared to be estradiol (Fig. 2. A, B, C and D). Spots for pure estradiol are also shown in the same figure (F, G).

Fixation with the ammonia-silver nitrate reagent produced from the hydrolyzed samples spots characteristic for glucuronic acid (Fig. 3. E and F).

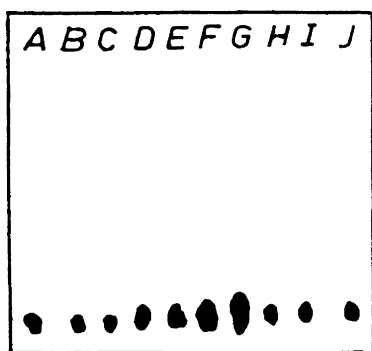


Fig. 1. Unconjugated estradiol. A, B, C, D, H, I, J spots of estradiol left unconjugated during the incubation (in the benzene-alcohol extract). Stained with Fast Black Salt K. G. control solution. E with 100 μg and F with 150 μg of estradiol.

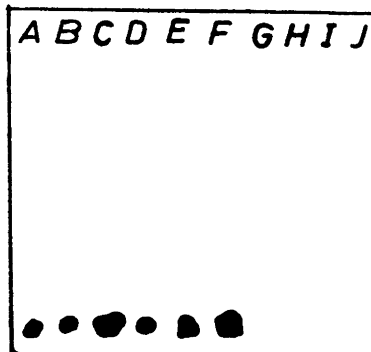


Fig. 2. Estradiol glucuronide. No dark spots characteristic for free estradiol could be obtained from the nonhydrolyzed ethylacetate residue after staining with Fast Black Salt K. (G, H, I, J). Free estradiol. A, B, C, D free estradiol spots after hydrolysis with β -glucuronidase. E and F obtained with 100 μg and 150 μg estradiol.

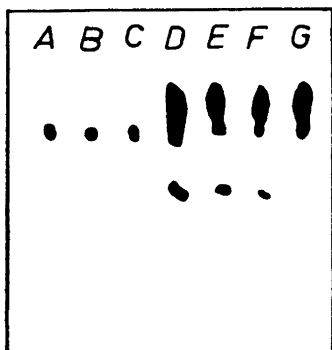


Fig. 3. Free glucuronic acid. E, F, G obtained by hydrolysis with β -glucuronidase; staining with the AgNO_3 -ammonia reagent. A, B, C nonhydrolyzed samples. D with 100 μg of glucuronic acid.

A spot developed from pure glucuronic acid is shown in the same picture (D). It is to be noted that the spot obtained from the hydrolyzed samples lies higher on the paper than that of pure glucuronic acid. Since this observation was familiar from our previous trials 50 μg pure glucuronic acid was added to the samples of the nonhydrolyzed residue. After the development of the chromatogram the dark spot (Fig. 3. G) appeared at the same point as in the original hydrolyzed samples (Fig. 3. E and F).

Since the spots are formed also with pure glucuronic acid the compound indicated by them apparently is formed during the run. They do not correspond to the R_F -values obtained with glucuronic acid lactone. One possibility is that they are esters formed from glucuronic acid and butanol. The presence of these additional spots does not invalidate the fact that spots representing free glucuronic acid are also produced by hydrolysis of the ethylacetate extract.

Unbound estradiol. The part of the estradiol which remained unconjugated during the incubation and which was extracted with the benzene alcohol extracts appeared very nicely as violet spots in the chromatograms (Fig. 1, A, B, C, D, H, I, J). These were, however, strikingly smaller and weaker than those in the control solutions (Fig. 1, G) in which the incubation procedures were made without any tissue slices. The comparison of spots A, B, C, D, H, I, J with G indicates that pure estradiol has been bound during the incubation. Spots E and F correspond to 100 μg and 150 μg pure estradiol.

Tissue specimens. In order to secure that no agents reacting in the chromatogram similar to estradiol were excreted from the tissue itself during the incubation the following control analyses were performed. Tissue slices were incubated in the usual manner but without the presence of estradiol. The chromatograms produced after this revealed no such spots either from the alcohol-benzene extracts, from the unhydrolyzed samples or from the hydrolysed samples. As a control 100 μg of pure estradiol was run on the same paper.

DISCUSSION

These experimental results indicate that the rat duodenum is capable to conjugate estradiol to the corresponding glucuronide *in vitro*. This conjugate can be hydrolyzed after extraction from acid incubation medium with ethylacetate. These components produced by hydrolysis are estradiol and glucuronic acid. So far these studies have demonstrated only the qualitative formation of estradiol glucuronide. The incubation solution contained 0.9 μmole of estradiol per 8—10 mg of tissue dry weight and a portion of the estradiol has apparently remained unconjugated. Although the exact amounts will be calculated in our later quantitative studies, it seems likely that perhaps some 40—50 % of the estradiol are bound to glucuronic acid. It is worth mentioning that the incubation was performed in a solution in which also sulphate ions were present. It is therefore possible that in addition to the glucuronide conjugation the estradiol has also reacted with sulphate.

These observations are taken as a further evidence to support the previous studies of Hartiala (1954 and 1955) in which *o*-aminophenol under similar conditions as above — with the exception that the incubation solution did not contain sulphate ions — was found to yield a conjugate similar to the *o*-aminophenolglucuronide produced by the liver.

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