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Duodenal Glucuronide Synthesis

IV. Studies of Steroidglucuronide Conjugation by the Duodenal Mucosa in Rat

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Steroid hormones were incubated in the presence of rat duodena slices. Paper chromatographic analyses were then performed on the ethylacetate extracts of the incubation mixture. These extracts revealed no free steroids and also no spots indicating glucuronic acid. Treatment of the ethylacetate evaporate with β-glucuronidase resulted in the liberation of free steroids and glucuronic acid when estrone, estradiol, estriol, equiline and stilbestrol were tested. No such glucuronide conjugates were identified for progesterone, pregnandiol, androsterone or testosterone. A phenolic hydroxyl at 3 C is a common feature of the former steroid group. None of the unconjugated steroids had this characteristic.

In our previous qualitative and quantitative studies we have identified estradiol glucuronide as a conjugation product formed by rat duodenal mucosa [1,2]. In the present work a number of other steroids have been subjected to similar analyses.

MATERIAL AND METHODS

Albino rats (Wistar) were used also in these experiments.

Steroids. Estriol, estrone, equiline, stilbestrol, progesterone, pregnandiol, androsterone and testosterone propionate were used. These were dissolved in 2 ml propyleneglycol [5] in the following amounts: 5.0 mg, 4.8 mg, 4.7 mg, 4.9 mg, 5.6 mg, 4.7 mg, 5.2 mg, 6.2 mg.

Incubation. The incubation of free steroids in the Warburg apparatus was performed as in the earlier studies [3,4]. The Krebs Ringer incubation solution 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 steroid propyleneglycol solution (0.9 μmole steroid) and after this the mucosal tissue specimens (dry weight 8–10 mg). The gas phase was 5 % carbon dioxide in oxygen.

Sampling. After incubation the contents of 2 Warburg flasks were combined to make one sample for further analyses (= 6 ml). The tissue slices were washed with 2 ml Ringer solution (6 ml + 2 ml) after being removed from the flasks.

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

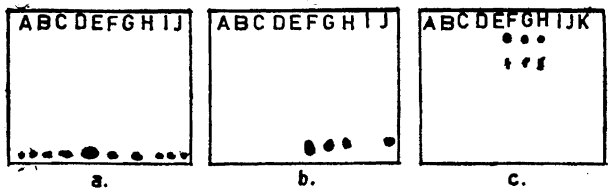

Fig. 1. Estrone. a. Unconjugated estrone. Positive spots (A, B, C, and D) for estrone present after the incubation of estrone with the tissue. The spots are, however, weaker than those obtained from the same incubation mixture without the tissue (G, H, I, and J). Control spots E (150 μg) and F (100 μg) pure estrone.

b. Estrone glucuronide acid and estrone liberated by hydrolysis. Unhydrolyzed samples show negative spots for free estrone (A, B, C, D, and E). Hydrolysis with β-glucuronidase results in liberation of free estrone (G, H, and J). Control, F, 150 μg estrone.

c. Estrone glucuronide and glucuronic acid liberated by hydrolysis. Unhydrolyzed samples give negative spots A, B, C, D, and E for free glucuronic acid. From the hydrolyzed samples (G, H, I, J, and K) G and H show positive spots for free glucuronic acid. F is obtained with pure glucuronic acid (150 μg). The spots which appear below the actual glucuronic acid spots are discussed in the text.

Extraction of free steroids with benzene, was carried out as in our earlier work [1,2].

Extraction of steroid glucuronides with ethylacetate from the acidified (2 N HCl) water phase was carried out as in the earlier work [1,2].

Hydrolysis of the conjugate was performed with β-glucuronidase (Worthington Biochem. Corp.) as in the previous papers [1,2]. Reextraction of the free steroids and glucuronic acid liberated by hydrolysis was also carried out as in the earlier work [1,2].

Paper chromatography. 1 ml of the solvent (ethylacetate/benzene) was added to each residue used for the chromatographic analyses. A mixture of butanol, glacial acetic acid and water was used as an irrigant; time 24 h, paper Whatman No. 1. Estriol, estrone, equiline, stilbestrol and pregnandiol spots were identified on the paper by a saturated aqueous solution of *p*-nitrobenzene-azodimethoxyaniline (Fast Black Salt, K. Heftman 1950 [6]). Stilbestrol gives a light brown spot on blue whereas the others give a blue red spot. Androsterone, testosterone and progesterone were identified with *m*-dinitrobenzene (1 vol. 2 % *m*-dinitrobenzene in alcohol + 1 vol. 2.5 N KOH in alcohol [7]). Glucuronic acid was identified with the $AgNO_3$-ammonia reagent (1 vol. 0.1 N $AgNO_3$, 1 vol. 5.0 N NH_3-solution + 1 vol. 2 N NaOH).

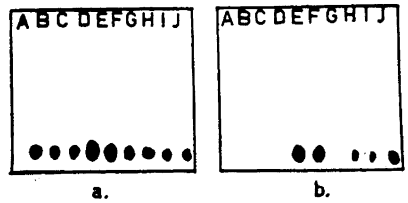

Fig. 2. Estriol. a. Unconjugated estriol. B, C, D, I, and J indicate free estriol present after incubation with the tissue slices. A is negative for some unknown reason. G and H, control runs made from the incubation mixture without the tissue. E and F pure estradiol (150 and 100 μg).

b. Estriol glucuronide acid and estriol liberated by hydrolysis. A, B, C, and D refer to the free estriol in the unhydrolyzed samples. H, I, and J, estriol liberated by hydrolysis. G is negative for some unknown reason. E and F, pure estriol (150 and 100 μg). From the hydrolyzed samples free glucuronic acid spots were never found. This is discussed in the text.

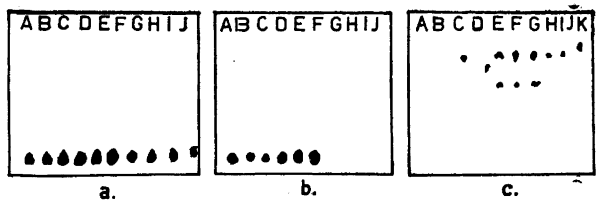

Fig. 3. Equiline. a. Unconjugated equiline. A, B, G, H, I, and J, free equiline present after incubation with the tissue slices. C and D, after incubation without the tissue. E and F, pure equiline (100 and 150 μg).

b. Unhydrolyzed equiline glucuronide and free equiline liberated by hydrolysis. G, H, I, and J refer to free equiline in the unhydrolyzed samples. A, B, C, and D free equiline after the hydrolysis. E and F pure equiline (100 and 150 μg).

c. Glucuronic acid liberated by hydrolysis. A, B, C, and E indicate the absence of free glucuronic acid before the hydrolysis. The positive spot D indicates that some liberation might take place without β-glucuronidase. H, I, J, and K, the same after enzymatic hydrolysis. F and G, pure glucuronic acid. Other spots seen in the pixture are discussed in the text.

RESULTS

In the following presentation the chromatograms of each of the steroids are given in a group. The first (left) picture of each group, a, indicates the spots due to steroids left unconjugated during the incubation. The second picture, b, indicates the results before and after hydrolysis of the conjugated steroids with β-glucuronidase. In this picture the absence of spots on the chromatograms of the unhydrolyzed steroid glucuronides or, in the cases in wich no glucuronide formation has occurred, the total absence of steroid spots is indicated. In the third picture, c, are indicated the glucuronic acid spots liberated during the hydrolysis. This picture, c, is not presented in Figs. 5—8, corresponding to experiments in which free glucuronic acid could never be demonstrated.

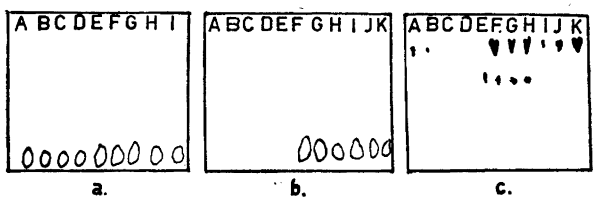

Fig. 4. Stilbestrol. a. Unconjugated stilbestrol. A, B, C, D, H, and I, free stilbestrol still present after incubation with the tissue slices. F and G, the same after incubation without the tissue. E, pure stilbestrol (150 μg).

b. Stilbestrol glucuronide and free stilbestrol liberated by hydrolysis. A, B, C, D, and E indicate negative spots for free stilbestrol before the hydrolysis. H, I, J, and K, free stilbestrol liberated by hydrolysis. F and G, pure stilbestrol (100 and 150 μg).

c. Glucuronic acid liberated by hydrolysis. A, B, C, D, and E, free glucuronic acid before the hydrolysis. Weak spots at A and B indicate some free glucuronic acid. G, H, I, J, and K the same after enzymatic hydrolysis. F, pure glucuronic acid (150 μg). The other spots present on the chromatogram are discussed in the text.

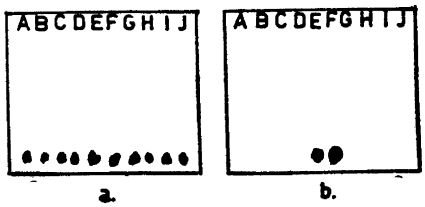

Fig. 5. Progesterone. a. Unconjugated progesterone. A, B, C, D, H, I, and J, free progesterone present after incubation with the tissue slices. E, control incubation without the tissue. F and G, pure progesterone (100 μg and 150 μg).

b. Absence of free progesterone in the unhydrolyzed and hydrolyzed samples. A, B, C, and D, before hydrolysis and G, H, I, and J, after hydrolysis with β-glucuronidase. E and F, pure progesterone (100 and 50 μg). The results indicate no glucuronide conjugation.

Positive results indicating glucuronide formation from the above listed estrogenic substances were obtained with

estrone	in	4	out	of	6	rats
estradiol	in	5	»	»	6	»
estriol	in	4	»	»	6	»
equiline	in	5	»	»	6	»
stilbestrol	in	6	»	»	6	»

Taking into consideration the size and color intensity of the different spots in the chromatogram stainings, it would appear that the degree of conjugation is not the same for the different estrogens. Using these indications as a rough estimate for the degree of conjugation, the following preliminary list can be given:

estrone	+
estradiol	+++ (reported in our previous paper [1,2])
estriol	+
equiline	++
stilbestrol	+++

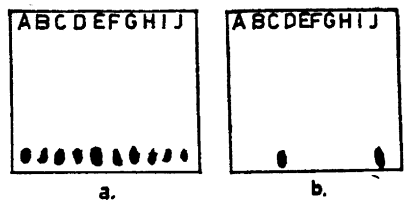

Fig. 6. Pregnandiol. a. Unconjugated pregnandiol. A, B, C, D, G, and H, free pregnandiol before incubation with the tissue slices. I and J, incubation without the tissue. E and F, pure pregnandiol (200 and 150 μg).

b. Absence of free pregnandiol in the hydrolyzed and unhydrolyzed specimens. A, B, C, before hydrolysis and E, F, G, H, and I, the hydrolyzed samples. D and J, pure pregnandiol (200 and 250 μg). The results indicate no glucuronide conjugation.

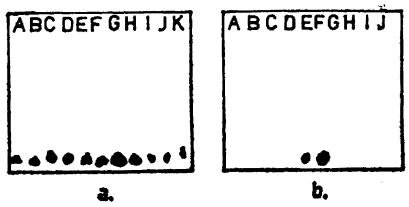

Fig. 7. Androsterone. a. Unconjugated androsterone. A, B, C, D, I, J, and K, free steroid present in the samples after incubation with the tissue slices. E and F, the same from the incubation mixture without the tissue. G and H, pure androsterone (150 and 100 μg).

b. Absence of free androsterone in the hydrolyzed and unhydrolyzed samples. A, B, C, and D, before hydrolysis and G, H, I, and J, after enzymatic hydrolysis. All spots are negative. E and F, pure androsterone (50 and 100 μg).

DISCUSSION

The chromatographic identification of the free and pure steroids used in these experiments has not met any difficulties. The presence of such spots on the chromatograms can also be taken as evidence of the reliability of the applied extraction procedures. On the other hand it occasionally appeared to be rather difficult to show free glucuronic acid on the chromatograms. In the case of estradiol, hydrolysis of the extract always resulted in the appearance of free glucuronic acid [1,2]. On the other hand glucuronic acid formation was detected only two or three times on 6 experiments with the other estrogens and never with estriol. The conjugate formation of the latter, as judged by the size and intensity of the free steroid spots of the conjugates in the different

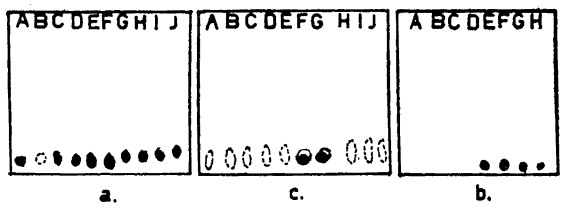

Fig. 8. Testosterone. a. Unconjugated testosterone. A, B, G, H, I, and J, free testosterone present after incubation with the tissue slices. C and D, samples incubated without the tissue. E and F, pure testosterone (150 and 100 μg). A positive spot was obtained for only one out of 6 animals.

b. Free testosterone in the hydrolyzed and unhydrolyzed specimens. A, B, C, and D negative spots for free testosterone in the unhydrolyzed samples. G and H, weak positive spots for free testosterone after hydrolysis. E and F, pure testosterone (150 and 100 μg).

c. In order to confirm the results obtained in the previous chromatograms the experiments were repeated three times with twice the amounts of solutions as used before. In the unhydrolyzed samples (A, B, C, D, and E) and in the hydrolyzed samples (H, I, and J) prolonged spots distinctly different from those given by pure testosterone (F and G, 150 and 100 μg) were found. These are discussed in the text. The results would indicate no glucuronide conjugation for the androgens.

chromatograms, is very small. It could be that the amount of glucuronic acid present is too small to give a positive spot on the paper. This possibility is enhanced by the fact that the experimental conditions do not eliminate the possibility of bacterial decomposition. This could reduce the amount of glucuronic acid present in the final steps of the analyses. This point was proved in our own experiments when it was found that free glucuronic acid added to the solutions under the same conditions as in the hydrolysis experiments disappeared completely in few days.

Extra spots are occasionally seen together with the glucuronic acid spots which arise from the apparent sensitivity of the $AgNO_3$ reagent towards various reducing substances. The small spots seen in several pictures beneath the glucuronic acid spots are identified as the lactone formed from the acid during the chromatographic procedure. These spots are also present when pure glucuronic acid alone is used.

The large, longish spots obtained with large amounts of the hydrolyzed and unhydrolyzed samples in connection with the repeated experiments with testosterone could not be more exactly identified due to lack of available pure testosterone conjugates. Since these spots stained with the same reagents as testosterone they might have an androgen molecular structure. The form of these spots would imply some kind of a salt compound. The possibility of a sulphate conjugate must also be considered. It may be pointed out that the tissue itself never interfered with the chromatographic analyses. Analyses of incubation mixture which contained tissue slices but not steroids never gave positive staining for spots which could erroneously be taken as steroid or glucuronic acid spots.

Finally, one interesting point can be stressed. If the interpretation of our results is correct, it would appear that glucuronide conjugation of substances possessing sexhormone activity is of a rather selective nature. Whereas the estrogens are conjugated, neither progesterone nor its metabolic product pregnandiol, nor the androgens are conjugated in this form. Examination of the molecular structure of these different substances reveals one interesting similarity of the glucuronide conjugated steroids which separates them from the others. They all have a phenolic hydroxyl group in the 3 C position. All of the compounds found to be unconjugated to a glucuronide lack this characteristic. It may be pointed out that in our previous studies indications of phenolphthalein and cinchophen glucuronide conjugation by the intestinal mucosa were found. The same molecular specificity also holds for these substances. The interpretation of this observation becomes however, rather complicated and difficult since recently a similar molecular characteristic, namely a phenolic hydroxyl at 3 C, has been found to belong to those steroids undergoing a sulphate conjugation[8].

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