however, the crude state of the enzyme has precluded serious characterization efforts.

References

Alberty, R. A. (1953), *J. Am. Chem. Soc. 75*, 1928.
Bowers, A., Halsall, T. G., Jones, E. R. H., and Lemin, A. J. (1953), *J. Am. Chem. Soc. 75*, 2548.
Burstein, S. H., and Ringold, H. J. (1964), *J. Am. Chem. Soc. 86*, 4952.
Dalziel, K. (1957), *Acta Chem. Scand. 11*, 1706.
Hayano, M., Ringold, H. J., Stefanovic, V., Gut, M., and Dorfman, R. I. (1961), *Biochem. Biophys. Res. Commun. 4*, 454.
Iriarte, J., Rosenkranz, G., and Sondheimer, F. (1955), *J. Org. Chem. 20*, 542.
Jones, R. N., and Cole, A. R. H. (1952), *J. Am. Chem. Soc. 74*, 5648.
Jones, R. N., Nolin, B., and Roberts, G. (1955), *J. Am. Chem. Soc. 77*, 6331.
Levy, H. R., and Talalay, P. (1959), *J. Biol. Chem. 234*, 2014.
Malhotra, S. K., and Ringold, H. J. (1965), *J. Am. Chem. Soc. 87*, 3228.
Massey, V., and Veeger, C. (1963), *Ann. Rev. Biochem. 32*, 579.
Ringold, H. J., Gut, M., Hayano, M., and Turner, A. (1962), *Tetrahedron Letters 18*, 835.
Ringold, H. J., Hayano, M., and Stefanovic, V. (1963), *J. Biol. Chem. 238*, 1960.
Ringold, H. J., and Turner, A. (1962), *Chem. Ind. (London)*, 211.
Stefanovic, V., Hayano, M., and Dorfman, R. I. (1963), *Biochim. Biophys. Acta 71*, 429.
Swain, C. G., Wiles, R. A., and Bader, R. F. W. (1961), *J. Am. Chem. Soc. 83*, 1945.

The Formation of Estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide by Rabbit Liver Homogenate*

Helmut Jirku† and Donald S. Layne

ABSTRACT: When 17β-estradiol-6,7-^{3}H was incubated with rabbit liver homogenates at pH 7.6 with uridine diphosphate *N*-acetylglucosamine in the presence of uridine diphosphate glucosiduronic acid, estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide was formed in amounts equivalent to about 10% of the total conjugated radioactivity. The double glycoside was identified by countercurrent distribution and then treated with β-glucuronidase. The estradiol-17α-*N*-acetylglucosaminide thus formed was identified with an authentic sample of this material by countercurrent, chromatographic, and isotope techniques. When estradiol-6,7-^{3}H and uridine diphosphate *N*-acetylglucosamine were incubated with liver homogenate in the absence of uridine diphosphate glucosiduronic acid, no transfer of *N*-acetylglucosamine to estradiol took place unless a prior incubation of the steroid and homogenate with uridine diphosphate glucosiduronic acid has been carried out. The results establish the presence in rabbit liver of a mechanism for the transfer of *N*-acetylglucosamine to the 17α-hydroxyl of estradiol and strongly indicate that the receptor is not the free steroid but the 3-glucuronoside. No evidence was found for the transfer of *N*-acetylglucosamine to the 17β-hydroxyl of estradiol.

The transfer of glucosiduronic acid from uridine diphosphate glucosiduronic acid (UDP-glucosiduronic acid[1]) to phenolic steroids by liver microsomal systems was indicated by the preliminary results of Isselbacher (1956). Smith and Breuer (1963) reported that estrone-3-glucuronoside is formed by the incubation of estrone and UDP-glucosiduronic acid with washed rabbit liver microsomes. Recent work (Layne *et al.*, 1964; Layne, 1965) has shown that much of the 17α-estradiol excreted in rabbit urine is in the form of the 3-glucuronoside-17α-*N*-acetylglucosaminide. This indicates that the rabbit possesses an enzyme system capable of transferring *N*-acetylglucosamine to the estrogen molecule. The failure to find evidence of the excretion of estrogen conjugates with glucosiduronic acid at position 17 or with *N*-acetylglucosamine at position 3 suggested that the two conjugating sites on 17α-estradiol might exhibit a high degree of specificity for the sugars. It also

* From the Worcester Foundation for Experimental Biology, Shrewsbury, Mass. *Received June 14, 1965; revised July 28, 1965.* Supported in part by grants (AM-06294 and AM-3419) from the National Institute of Arthritis and Metabolic Diseases. Presented in part at the 49th meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1965.

† Trainee in the Steroid Training Program supported by a grant (T4 CA-5001) from the National Cancer Institute.

[1] Abbreviation used in this work: UDP, uridine diphosphate.

seemed possible that the conjugation with one sugar molecule might require prior conjugation with the other. This paper presents evidence that rabbit liver contains an enzyme which transfers *N*-acetylglucosamine from uridine diphosphate *N*-acetylglucosamine (UDP-*N*-acetylglucosamine) to the 17α-hydroxyl of estradiol and that the reaction appears to require the prior formation of the 3-glucuronoside.

Experimental Procedure and Results

Materials. Solvents were purified and stored as previously described (Layne *et al.*, 1964). 17α-Estradiol-16-^{14}C was obtained in previous experiments from the urine of rabbits injected with estrone-16-^{14}C (Layne, 1965). The material was purified by countercurrent distribution in methanol–water–carbon tetrachloride–chloroform (7:3:6:4) and had a specific activity of 4.6×10^{-5} μcurie/μg. 17β-Estradiol-6,7-^{3}H of specific activity 50 μcuries/μg was prepared in this laboratory (Uskokovic and Gut, 1957) and was diluted with carrier 17β-estradiol before use. All radioactive and reference steroids were pure as judged by paper chromatography. UDP-*N*-acetylglucosamine and UDP-glucosiduronic acid were obtained from Sigma Chemical Co.

Preparation of Homogenates. Rabbits were killed by neck fracture, and the liver was immediately excised. A portion (20 g) of the tissue was homogenized in a Waring blendor with 80 ml of 0.15 M potassium chloride.

Preliminary Experiments. Liver homogenate (from 0.5 to 3.0 ml) and a similar volume of 0.15 M phosphate buffer, pH 7.6, were added to 15-ml centrifuge tubes containing from 0.1 to 0.6 μmole of radioactive steroid dissolved in from 0.1 to 0.6 ml of propylene glycol. The UDP-glucosiduronic acid or UDP-*N*-acetylglucosamine was added in solution in a small volume of 0.15 M potassium chloride. A similar volume of the potassium chloride solution was added to the control tubes. The tubes were shaken in air in a Dubnoff incubator at 37° for 1 hr and were then placed in a boiling water bath for 1 min. A volume of chloroform equal to that of the aqueous layer was added, and the tubes were stoppered and shaken vigorously. The chloroform was removed, the extraction was repeated, and the amount of radioactivity in the combined chloroform extracts and in the aqueous layer was determined by liquid scintillation counting. The conjugates were poorly soluble in toluene so that to obtain accurate counts of the aqueous layer it was necessary to add a 0.5-ml aliquot to 6 ml of ethanol and 10 ml of toluene solution of liquid scintillator. A correction for quenching was made by the use of radioactive toluene as internal standard. The procedures for counting and for determination of quenching were those described by Flood *et al.* (1961).

Provided that the amount of steroid incubated was not less than 0.6 μmole/ml of homogenate, adsorption of the steroid by precipitated protein was relatively small, and total recoveries of added radioactivity were consistently from 82 to 87% for control samples and better than 70% when conjugation had taken place. The percentage of the added radioactivity which was not extracted into chloroform, as compared to a blank sample, was used as a preliminary measure of the extent of conjugation.

Little or no conjugation of estrogen by the liver homogenate was observed in the absence of added nucleotides. When UDP-glucosiduronic acid was added conjugation was always observed, and the amount of steroid conjugated ranged from 0.02 to 0.04 μmole/ml of homogenate. When UDP-*N*-acetylglucosamine was added, there was no significant evidence of conjugation. Elimination of propylene glycol from the incubation medium did not affect this result. In some, but not all, experiments, evidence was obtained of a small amount of conjugation when the homogenate and steroid were pre-incubated for 30 min before addition of UDP-*N*-acetylglucosamine. No chromatographic evidence for the presence of estradiol-17α-*N*-acetylglucosaminide in these incubates could be found.

When UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine were added together to the incubation medium, a marked decrease in the amount of chloroform-extractable steroid radioactivity, as compared to the value obtained on incubation with the same amount of UDP-glucosiduronic acid in the absence of UDP-*N*-acetylglucosamine, was often observed. This effect, which appears to be an enhancement of glucuronidation in the presence of UDP-*N*-acetylglucosamine, has been studied by Pogell and Leloir (1961). In our experiments the magnitude of the enhancement varied greatly with the incubation conditions and with the relative amounts of the two nucleotides present.

No differences in the pattern of conjugation were observed between the results obtained in preliminary experiments with 17β-estradiol and those in which 17α-estradiol was used. Because of the very limited amount of radioactive 17α-estradiol available to us and because of evidence that 17α-estradiol and 17β-estradiol are interconverted by rabbit liver homogenate (Breuer and Pangels, 1960) further experiments were carried out with the 17β isomer.

Chromatographic Examination of the Conjugates Formed by Liver Homogenate. The aqueous layers from some of the preliminary experiments were adjusted to pH 2.0 and extracted with butanol. The butanol extracts were examined by thin-layer chromatography on silica gel G in chloroform–ethanol (7:3) and on Ecteola cellulose in 1.5 M acetate buffer, pH 4.5 (Oertel *et al.*, 1964). Large amounts of radioactive material were always found at the R_F of 17α-estradiol-3-glucuronoside in samples in which UDP-glucosiduronic acid had been present in the incubation. Varying amounts of radioactivity, which might have been due either to diglucuronides or to double glycoside, were found in samples from incubations with both UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine. In neither system was there evidence of the presence of estradiol-17α-*N*-acetylglucosaminide in any of the incubates.

Effect of Incubation with UDP-N-acetylglucosamine after Incubation with Increasing Amounts of UDP-

glucosiduronic Acid. A solution of 0.3 μmole (100,000 cpm) of 17β-estradiol-6,7-^{3}H in 0.2 ml of propylene glycol was added to each of 10 tubes containing 2.0 ml of liver homogenate and 1.5 ml of buffer. The tubes were divided into pairs, and to each pair a different amount of UDP-glucosiduronic acid, over a range from 0 to 0.6 μmole, was added in 0.8 ml of 0.15 M potassium chloride. The tubes were incubated at 37° for 1 hr. To one tube of each pair 1.2 μmoles of UDP-*N*-acetylglucosamine was added in 0.1 ml of 0.15 M potassium chloride. An equal volume of the potassium chloride solution was added to the other tube of each pair.

The tubes were re-incubated for 1 hr at 37°, and the contents of each were extracted three times with an equal volume of chloroform. An aliquot of 0.1 ml of the water phase was assayed for radioactivity. In each case, the amount of radioactivity found in the water phase of the tubes to which UDP-*N*-acetylglucosamine had been added was slightly but significantly greater than that in the companion tube which had received the same amount of UDP-glucosiduronic acid but no UDP-*N*-acetylglucosamine.

Two further aliquots, each of 0.2 ml, were removed from the aqueous phase of each incubation tube, and each aliquot was added to a tube containing 10 ml of 0.5 M acetate buffer, pH 5.0. To one sample from each incubation tube 2000 units of β-glucuronidase (Ketodase, Warner-Chilcott) was added. The other tube served as a control. All the tubes were incubated at 37° for 16 hr, and the contents were first extracted twice with chloroform and then once with an equal volume of ethyl acetate. The counts in the ethyl acetate fraction therefore represented material which could be extracted from the aqueous phase at pH 5.0 by ethyl acetate but not by chloroform. Layne *et al.* (1964) have shown that estradiol-17α-*N*-acetylglucosaminide possesses this characteristic.

Table I shows that the radioactivity in the ethyl acetate extract after enzyme treatment was consistently higher in samples from liver incubations of radioactive steroid with both UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine than in samples from incubations performed with UDP-glucosiduronic acid alone. This difference can be attributed to the formation of estradiol-3-glucuronoside-17-*N*-acetylglucosaminide, which yields estradiol-17-*N*-acetylglucosaminide on treatment with Ketodase.

The ethyl acetate extracts from the enzyme-treated samples from tubes incubated with both UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine were pooled. The corresponding extracts from tubes incubated with UDP-glucosiduronic acid only were also pooled. The pooled extracts were chromatographed on thin-layer silica gel G in chloroform–ethanol (7:3). The extract from the incubate with both nucleotides contained major amounts of a radioactive material which had the same R_F as estradiol-17α-*N*-acetylglucosaminide. Very small amounts of radioactivity were observed at this R_F in the extract from the control tubes.

Evidence for Transfer of N-Acetylglucosamine to Estradiol Glucuronoside. Approximately 5 mg of 17β-estradiol-7-^{3}H of specific activity 100 μc/mg was incubated for 1 hr with 140 ml of liver homogenate in the presence of 21 mg of UDP-glucuronic acid. The incubate was thoroughly extracted with chloroform, and three volumes of ethanol was added to the aqueous layer. The precipitate was filtered, and the radioactivity in the filtrate was distributed twice through 99 transfers in ethyl acetate–butanol–water (3:1:4). The radioactivity in the peak fraction from this distribution was largely released as free steroid by treatment with Ketodase. The free steroid was shown on chromatograms to consist mainly of 17β-estradiol, although some 17α-estradiol and estrone were also present.

To each of ten 50-ml erlenmeyer flasks was added 8 ml of liver homogenate, 8 ml of phosphate buffer, pH 7.6, and 2.4 μmoles of UDP-*N*-acetylglucosamine. To one flask an aliquot of the crude glucuronoside mixture prepared above, equivalent to 1.2 μmoles calculated as estradiol-3-glucuronoside, was added in 0.8 ml of propylene glycol. No UDP-glucosiduronic acid was added to this flask. Each of the other flasks received 1.2 μmoles of 17β-estradiol-7-^{3}H in 0.8 ml of propylene

TABLE I: Radioactivity Extractable at pH 5.0 by Ethyl Acetate but not by Chloroform after Ketodase Treatment of the Conjugates Formed by Incubation of 0.3 μmole of 17β-Estradiol-7-^{3}H with Rabbit Liver Homogenates in the Presence of Added Nucleotides.

Sample	μmoles UDP-Glucosidur-onic acid/ 4.5 ml Incubate	μmoles UDP-*N*-Acetyl-glucos-amine/ 4.5 ml Incubate[a]	Conjugated Radio-activity Extracted by Ethyl Acetate after Ketodase Treatment (%)	Conjugated Radio-activity as Acetyl-glucos-aminide[b] (%)
1	0.0	0.0	18.4	..
2	0.0	1.2	14.3	0.0
3	0.12	0.0	10.5	..
4	0.12	1.2	21.0	11.5
5	0.3	0.0	7.1	..
6	0.3	1.2	26.3	19.2
7	0.45	0.0	5.3	..
8	0.45	1.2	15.5	10.2
9	0.6	0.0	3.4	..
10	0.6	1.2	14.4	11.0

[a] The steroid and UDP-glucosiduronic acid were incubated for 1 hr with the homogenate, UDP-*N*-acetylglucosamine was added, and the incubation was continued for another hour. [b] Determined from values in previous column as difference between sample pairs incubated with the same amount of UDP-glucosiduronic acid, with and without UDP-*N*-acetylglucosamine.

glycol and 1.8 μmoles of UDP-glucosiduronic acid. The flasks were incubated at 37° for 1 hr.

An aliquot of 0.1 ml of the incubate from the flask containing pre-formed glucuronoside and a similar aliquot from one of the other flasks were placed in separate tubes containing 5 ml of 0.5 M acetate buffer, pH 5.0. After the addition of 2500 units of Ketodase, the tubes were incubated at 37° for 16 hr. The contents of each tube was extracted three times with 10 ml of chloroform to remove free steroid and then three times with 10 ml of ethyl acetate to remove estradiol-17-*N*-acetylglucosaminide (Layne *et al.*, 1964). The ethyl acetate extracts were evaporated to dryness and chromatographed on thin-layer silica gel G in chloroform–ethanol (3:7) in parallel with authentic estradiol-17α-*N*-acetylglucosaminide (Layne *et al.*, 1964). The chromatogram was sprayed with Folin–Ciocalteu reagent to locate the standard material and was then assayed for radioactivity by eluting small areas into vials for liquid scintillation counting (Arai *et al.*, 1962). Peaks of radioactivity were found at the R_F of the authentic estradiol-17α-*N*-acetylglucosaminide in the extracts from both incubates.

Identification of Estradiol-3-glucuronoside-17α-N-acetylglucosaminide. The contents of the incubation flasks from the experiment described above were pooled and extracted twice with 200 ml of chloroform. The residue was centrifuged and the aqueous layer was added to 1 liter of ethanol. After filtration the protein residue was washed with ethanol and the washings were added to the filtrate. After standing overnight, the ethanolic solution was filtered and the residue was washed with 100 ml of warm ethanol. The combined filtrate was evaporated to dryness and the resultant oil applied in 1 ml of ethanol to a 26 × 2 cm column of silica gel (Davison, 950 mesh). The column was eluted with increasing concentrations of ethanol in chloroform. A peak of radioactivity was eluted at an ethanol concentration of 10%, and further small amounts of radioactivity were eluted as the ethanol concentration was raised. Chromatographic examination showed that this material consisted of free steroid and some monoglucuronoside. The double conjugate was eluted, together with most of the monoglucuronoside, when methanol was substituted for ethanol. The resulting eluate was free of the propylene glycol used in the incubation. No evidence of the presence of estradiol-17-*N*-acetylglucosaminide was found on chromatographic examination of any of the eluates.

The polar eluate was distributed through 99 transfers in the countercurrent system ethyl acetate–butanol–water (5:3:8). The minor peak of radioactivity had a K value of 0.68 (peak A, Figure 1). The estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide characterized by Layne (1965) has an identical K value in this system. The major peak with K of 3.5 (peak B, Figure 1) was redistributed through 99 transfers in ethyl acetate–cyclohexane–ethanol–water, 7:3:3:7. No radioactivity was found at K = 0.39, which is the value given by estradiol-17α-*N*-acetylglucosaminide in this system (Layne *et al.*, 1964).

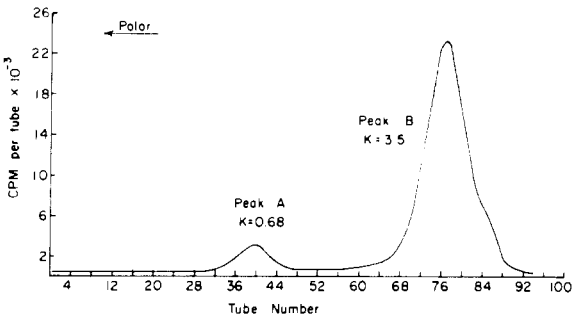

FIGURE 1: Countercurrent distribution in ethyl acetate–butanol–water (5:3:8) of the water-soluble radioactivity obtained by incubating 17β-estradiol-6,7-^{3}H with rabbit liver homogenate in the presence of both UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine.

The tubes containing peak A (Figure 1) were pooled. The residue was dissolved in 10 ml of 0.3 M acetate buffer, pH 5.0, and incubated at 37° for 24 hr with 5000 units of Ketodase in the presence of 10 mg of *N*-acetylglucosamine/ml to inhibit *N*-acetylglucosaminidase (Pugh *et al.*, 1957). The aqueous solution was then extracted three times with 10 ml of ethyl acetate, incubated with a further 5000 units of Ketodase for an additional 24 hr, and again extracted with ethyl acetate. The combined ethyl acetate extract contained 340,000 cpm of tritium. A small aliquot of the extract was chromatographed on thin-layer silica gel G in chloroform–ethanol (3:2), in parallel with authentic estradiol-17α-*N*-acetylglucosaminide. All of the radioactivity was located in the extract at the R_F of the authentic material.

The remainder of the ethyl acetate extract was dried and mixed with 13,780 cpm of 16-^{14}C-estradiol-17α-*N*-acetylglucosaminide of specific activity 19 cpm/μg (Layne *et al.*, 1964). This mixture was distributed through 99 transfers in ethyl acetate–cyclohexane–ethanol–water, 7:3:3:7. The major peaks of ^{3}H and of ^{14}C (Figure 2) were coincident at K = 0.39. A small amount of ^{3}H was found in the final fractions of the distribution. This material was chromatographically identical with 17α-estradiol, a small amount of which is liberated by Ketodase from the double glycoside (Layne *et al.*, 1964).

Aliquots from tubes 22, 28, and 34 in the distribution shown in Figure 2, which represented three different points in the peak of radioactivity corresponding to estradiol-17α-*N*-acetylglucosaminide, were assayed for ^{14}C and ^{3}H with corrections for quenching and for the contribution of each isotope to the assay of the other (Flood *et al.*, 1961). The ^{3}H/^{14}C ratio for each tube was calculated and the results were averaged. The tubes constituting the peak were pooled, and one-third of the residue was chromatographed on silica gel G in chloroform–ethanol, 7:3. The area corresponding to estradiol-17α-*N*-acetylglucosaminide was eluted. The ^{3}H/^{14}C ratio in an aliquot of the eluate was determined. The

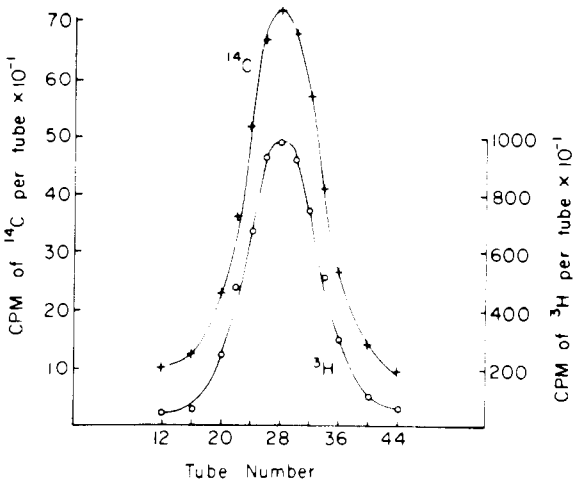

FIGURE 2: Countercurrent distribution in ethyl acetate–cyclohexane–ethanol–water (7:3:3:7) of a mixture of authentic estradiol-^{14}C-17α-*N*-acetylglucosaminide with the material produced by treatment with β-glucuronidase of the double glycoside of estradiol-^{3}H formed by rabbit liver homogenate.

remaining eluate was dried and treated overnight with 1 ml of acetic anhydride in 1 ml of pyridine and then evaporated to dryness. The acetylated material was partitioned between ethyl acetate and water, and the ethyl acetate extract was chromatographed on silica gel G in ethyl acetate–cyclohexane (2:1) (Layne *et al.*, 1964). A single peak of fully acetylated estradiol-17α-*N*-acetylglucosaminide was eluted. The $^{3}H/^{14}C$ ratio of the eluate was determined.

Another aliquot of one-third of the countercurrent peak (Figure 2) corresponding to estradiol-17α-*N*-acetylglucosaminide was incubated in 0.05 M citric acid at pH 4.2 for 24 hr with 100 mg of almond emulsin to remove the *N*-acetylglucosamine. The aqueous layer was extracted three times with chloroform, incubated for a further 24 hr, and again extracted. Aliquots of the chloroform layer were chromatographed on silica gel G in ethyl acetate–cyclohexane (3:7) and on formamide-impregnated paper in heptane–benzene (1:1). In each case the radioactivity was coincident with authentic 17α-estradiol, and there was no evidence of the presence of 17β-estradiol. The $^{3}H/^{14}C$ ratio of eluates of the radioactivity from both chromatographic systems was determined. These were similar (Table II) to the ratios obtained in the preceding steps of the identification.

Discussion

The results of the large-scale experiment in which 17β-estradiol-6,7-^{3}H was incubated with liver homogenate in the presence of both UDP-glucosiduronic acid and UDP-*N*-acetylglucosamine provide conclusive proof of the formation of estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide by rabbit liver.

The fact that incubation of 17β-estradiol leads to the formation of a glycoside in the 17α position is not surprising, since Breuer and Pangels (1960) have shown that 17β-estradiol is partially converted to the 17α isomer by rabbit liver homogenates. In our experiments, in which the ratio of steroid to liver tissue is high, the percentage conversion of the 17β to the 17α isomer is not large. Figure 1 indicates that the yield of the double glycoside (peak A) is small as compared to that of total glucuronoside (peak B), and it seems probable that epimerization of the 17-hydroxyl group to the α orientation is a prerequisite for formation of the *N*-acetylglucosaminide. This conclusion is in accord with the failure to detect any conjugation of the 17β-hydroxyl of estradiol with *N*-acetylglucosamine.

The results in Figure 1 indicate that only monoglucuronosides are present in peak B, since diglucuronosides would be more polar than the double glycoside in peak A. Since glucosiduronic acid can be readily added to the C-3 position (Smith and Breuer, 1963), it seems likely that the rabbit does not form either 17α- or 17β-glucuronosides of estradiol. In previous work on the urinary excretion products of estrone-^{14}C in the rabbit (Layne *et al.*, 1964; Layne, 1965), we have found that estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide accounts for about 80% of the excreted radioactivity, and no evidence has been found for the excretion of either estrone-3-glucuronoside or any other monoglucuronoside. These results, together with those of the present study, suggest that excretion of the estrogen molecule in the rabbit requires conjugation at both C-3 and C-17 and indicate that the absence of 17β-estradiol as a urinary excretion product may be due to the absence of a mechanism for conjugation of the 17β-hydroxyl group.

Pogell and Leloir (1961) have shown that addition of UDP-*N*-acetylglucosamine produces an increase in the transfer of glucosiduronic acid to phenols from UDP-glucosiduronic acid by liver microsomes. In their experiments this effect was somewhat variable and was shown to be partly due to inhibition of breakdown of UDP-glucosiduronic acid. The increased conversion of steroid to water-soluble products which often occurred

TABLE II: Ratio of $^{3}H/^{14}C$ in Mixture of Presumptive Estradiol-17α-*N*-acetylglucosaminide-6,7-^{3}H with Authentic Estradiol-17α-*N*-acetylglucosaminide-16-^{14}C and of the 17α-Estradiol Derived from This mixture.

Fraction and Treatment	$^{3}H/^{14}C$ Ratio
Estradiol-17α-*N*-acetylglucosaminide following	
Countercurrent distribution (Figure 2)	17.4
Chromatography on silica gel G	17.1
Acetylation and rechromatography	17.1
17α-Estradiol following	
Chromatography on silica gel G	17.1
Chromatography on paper	16.6

in the present experiments when both nucleotides were present, as compared to that which occurred when UDP-glucosiduronic acid was present alone, was largely due to enhanced formation of glucuronoside, since formation of the double glycoside would not greatly increase the partition coefficient of a monoglucuronoside between water and chloroform. However, the results in Table I show that after treatment of the water-soluble products with β-glucuronidase the tubes that had been incubated with both nucleotides contained a radioactive material which could be extracted with ethyl acetate and not with chloroform. The chromatographic identity of this material with estradiol-17α-*N*-acetylglucosaminide indicates that estradiol-3-glucuronoside-17α-*N*-acetylglucosaminide was present in these incubates. This provides strong evidence that the transfer of *N*-acetylglucosamine to the steroid is effected from UDP-*N*-acetylglucosamine.

The failure to demonstrate transfer of *N*-acetylglucosamine to estradiol from UDP-*N*-acetylglucosamine, except either in the presence of UDP-glucosiduronic acid or after pre-incubation of the liver tissue with steroid and UDP-glucosiduronic acid, suggests that this transfer requires the prior conjugation of the 3-hydroxyl group of the steroid with glucosiduronic acid. This indication is supported by the fact that, when a crude glucuronoside mixture was prepared, extracted from the liver tissue, and used as substrate in a new incubation with UDP-*N*-acetylglucosamine, significant transfer of *N*-acetylglucosamine to the pre-formed glucuronoside could be demonstrated. The failure to detect any evidence for the presence of a conjugate of estradiol with *N*-acetylglucosamine alone, either in chromatographic examination of the various incubates, in countercurrent examination of the products of the large-scale incubation, or in any of the previous work on rabbit urine (Layne *et al.*, 1964; Layne, 1965), strengthens the evidence that prior formation of the glucuronoside is necessary. Definitive proof of this point will require the at least partial purification of the *N*-acetylglucosamine transferase enzyme and the preparation of 17α-estradiol-3-glucuronoside for use as substrate.

Neither in this work nor in that on rabbit urine (Layne *et al.*, 1964; Layne, 1965) has any evidence been found of conjugates of estradiol with *N*-acetylglucosamine at position 3 or with glucosiduronic acid at position 17. This indicates that, in the rabbit, a high degree of specificity exists for the two sites on the estradiol molecule. This, together with the strong indication from the present work that the acceptor for *N*-acetylglucosamine is 17α-estradiol-3-glucuronoside, leads to speculation as to whether the conjugation of the estradiol molecule to these two sugars is solely for purposes of excretion. It is possible that this marked specificity in the attachment of the sugars to the two conjugating sites may be related to some effect of the estrogen on mucopolysaccharide in rabbit tissues.

Acknowledgments

The authors are indebted to Mrs. Katherine S. Whittemore for technical assistance.

References

Arai, K., Golab, T., Layne, D. S., and Pincus, G. (1962), *Endocrinology 71*, 639.

Breuer, H., and Pangels, G. (1960), *Acta Endocrinol. 33*, 532.

Flood, C., Layne, D. S., Ramcharan, S., Rossipal, E., Tait, J. F., and Tait, S. A. S. (1961), *Acta Endocrinol. 36*, 237.

Isselbacher, K. J. (1956), *Recent Prog. Hormone Res. 12*, *134*.

Layne, D. S. (1965), *Endocrinology 76*, 600.

Layne, D. S., Sheth, N. A., and Kirdani, R. Y. (1964), *J. Biol. Chem. 239*, 3221.

Oertel, G. W., Tornero, M. C., and Groot, K. (1964), *J. Chromatog. 14*, 509.

Pogell, B. M., and Leloir, L. F. (1961), *J. Biol. Chem. 236*, 293.

Pugh, D., Leaback, D. H., and Walker, P. G. (1957), *Biochem. J. 65*, 464.

Smith, E. R., and Breuer, H. (1963), *Biochem. J. 88*, 168.

Uskokovic, M., and Gut, M. (1957), *J. Org. Chem. 22*, 996.