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Transfer of *N*-Acetylglucosamine from Uridine Diphosphate *N*-Acetylglucosamine to 3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one by Human Adult and Fetal Kidney Homogenates*

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ABSTRACT: 3,15α-Dihydroxyestra-1,3,5 (10)-trien-17-one (15α-OHE_1) and its 3-sulfate are found in human body fluids principally as conjugates of *N*-acetylglucosamine. The biosynthesis of these novel conjugates was achieved. Human adult and fetal kidney homogenates fortified with uridine diphosphate *N*-acetylglucosamine converted [^3H]15α-OHE_1 into 3-hydroxy-17-oxoestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (15α-OHE_1GNAc). Hydrolysis by a pure preparation of jack bean meal β-*N*-acetylhexosaminidase yielded 15α-OHE_1, strongly suggesting the β configuration for the glycosidic bond. Reduction of 15α-OHE_1GNAc with $NaBH_4$ followed by methylation with diazomethane and subsequent acid hydrolysis yielded 3-methoxyestra-1,3,5(10)-triene-15α, 17β-diol, thereby establishing C-15 as the position of attachment of the sugar moiety. In a similar fashion 15α-OHE_1-3 sulfate was converted into 3-sulfato-17-oxoestra-1,3,-5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside. Attempts to demonstrate significant *N*-acetylglucosaminyl transferase activity in fresh human fetal liver and adult liver obtained post mortem have been unsuccessful.

3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one and estra-1,3,5(10)-triene-3,15α,17β-triol have been detected in fetal tissues and maternal urine in human pregnancy (Schwers *et al.*, 1965a,b,c; Knuppen *et al.*, 1965; Lisboa *et al.*, 1967), and in the bile and urine of a nonpregnant subject given labeled estrone sulfate (Jirku and Levitz, 1969). Of particular interest is that these steroids, 15α-OHE_1[1] and 15α-OHE_2, are excreted principally in the form of *N*-acetylglucosaminides. The *N*-acetylglucosaminides are found as single conjugates and as double conjugates, the second conjugating moiety being sulfate (Jirku and Levitz, 1969, 1970). Considering that only two other laboratories have isolated and identified steroid *N*-acetylglucosaminides (Layne *et al.*, 1964; Arcos and Lieberman, 1967), these conjugates are novel. The purposes of the present study were to see whether the biosynthesis of *N*-acetylglucosaminides of 15α-hydroxyestrogens could be

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[1] Abbreviations used are: 15α-OHE_1, 3,15α-dihydroxyestra-1,3,5-(10)-trien-17-one; 15α-OHE_1-3S, 3-sulfato-15α-hydroxyestra-1,3,5(10)-trien-17-one; 15α-OHE_1GNAc, 3-hydroxy-17-oxoestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside; 15α-OHE_1SGNAc, 3-sulfato-17-oxoestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside; 15α-OHE_2, estra-1,3,5(10)-triene-3,15α,17β-triol; 15α-OHE_2GNAc, 3,17β-dihydroxyestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside; 15α-OHE_2SGNAc, 3-sulfato-17β-hydroxyestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside; GNAc, *N*-acetylglucosaminide; SGNAc, sulfo-*N*-acetylglucosaminide; UDPGNAc, uridine diphosphate *N*-acetylglucosamine; HBV, holdback volume; TEAMS, triethylammonium sulfate.

TABLE I: Solvent Systems for Partition Chromatography on Celite.

System No.		
1	Stationary phase	13 ml of ethylene glycol in 26 g of Celite above 3 ml of H_2O in 10 g of Celite
	Mobile phase	700 ml of isooctane
	Gradient solvent	475 ml of ethyl acetate (gradient started at tube 4)
2	Stationary phase	Lower phase from isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–NH_4OH–H_2O (150:400:200:200:0.1:300)[a]
	Mobile phase	Upper phase from same equilibration
3	Stationary phase	Lower phase from benzene–ethyl acetate–methanol–H_2O (13:7:11:9)
	Mobile phase	Upper phase from benzene–methanol–H_2O (800:675:550)
	Gradient solvent	400 ml of ethyl acetate
4	Stationary phase	Upper phase from isooctane–ethyl acetate–1,2-dichloroethane–H_2O–0.6 M TEAMS[b] (2:10:2:6:18) (gradient started at tube 9)
	Mobile phase	Lower phase from isooctane–ethyl acetate–H_2O–0.6 M TEAMS (100:450:50:150)
	Gradient solvent	700 ml of 1,2-dichloroethane (5-ml cuts were taken)
5	Stationary phase	Upper phase from ethyl acetate–*n*-butyl alcohol–0.6 M TEAMS (400:400:100)
	Mobile phase	Lower phase from same equilibration
	Gradient solvent	Upper phase from *n*-butyl alcohol–0.6 M TEAMS (400:100)
6	Stationary phase	Upper phase from 1,2-dichloroethane–*tert*-butyl alcohol–H_2O–0.6 M TEAMS (450:200:300:300)
	Mobile phase	Lower phase from same equilibration
	Gradient solvent	400 ml of *tert*-butyl alcohol
7	Stationary phase	Lower phase from isooctane–benzene–ethyl acetate–methanol–H_2O (15:5:10:12:8)
	Mobile phase	Upper phase from isooctane–benzene–methanol–H_2O (600:200:600:500)
	Gradient solvent	300 ml of ethyl acetate
8	Stationary phase	Lower phase from isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–NH_4OH–H_2O (200:600:400:400:0.3:100)
	Mobile phase	Upper phase from same equilibration
9	Stationary phase	Upper phase from 1,2-dichloroethane–*tert*-butyl alcohol–H_2O (500:100:400)
	Mobile phase	Lower phase from same equilibration
	Gradient	400 ml of *tert*-butyl alcohol

[a] The values in parentheses represent the actual volumes (ml) used. [b] The use of TEAMS for chromatography of sulfate conjugates has been described by Mickan *et al.* (1969).

achieved in homogenates of adult and fetal kidney and liver and to elucidate the structure of the conjugates.

Experimental Section

Chromatography. Most separations were effected by chromatography on Celite. Acid-washed Celite 545 (Johns-Manville, Manville, N. J.) was washed twice with acetone and air-dried before use. Celite (36 g) was mixed with 18 ml of stationary phase and packed into a 2.0 × 45 cm column according to the directions of Siiteri (1963). The sample was dissolved in 1.5 ml of stationary phase, mixed with 3 g of Celite, and packed on top. The apparatus used for gradient elution was that described by Engel *et al.* (1961). Unless indicated otherwise, the gradient was started as soon as the first drop of solvent appeared at the bottom of the column, and 10-ml cuts were taken. The solvent systems are shown in Table I. The preparation of alumina and Sephadex columns has been described previously (Emerman *et al.*, 1967; Jirku and Levitz, 1969). Thin-layer chromatography was carried out on silica gel G. The solvent system was chloroform–ethanol (7:4, v/v), the plate being developed twice to effect the separation of 15α-OHE_1GNAc from 15α-OHE_1-3 sulfate.

Materials. [6,7-^3H]Estrone was purchased from Amersham/Searle and [16-^{14}C]estrone was synthesized in this laboratory (Levitz, 1953). Following chromatography in system 1 (estrone was eluted in HBV 4–5) they were converted, respectively, into [^3H]15α-OHE_1 (300 μCi/mg) and [^{14}C]15α-OHE_1 (11 μCi/mg) by fermentation in media of *Glomerella fusarioides* by an adaptation (Frey *et al.*, 1970) of the method of Laskin *et al.* (1964). The purity of the [^3H]15α-OHE_1 was demonstrated by the double isotope dilution technique. It was mixed with authentic [^{14}C]15α-OHE_1 obtained through the generosity of Dr. P. Diassi, Squibb Institute for Medical Research, and Dr. S. Solomon and the ^3H:^{14}C ratios were checked for constancy through several purification steps (Jirku *et al.*, 1967). 15α-OHE_2 was prepared by reducing 15α-OHE_1 with $NaBH_4$ and purified in system 3 (HBV 4). The 3-methyl ether of 15α-OHE_2 was prepared (Brown, 1955) and purified in system 1 (HBV 9). The 3-sulfate of [^3H]15α-OHE_1 was prepared (Dusza *et al.*, 1968) and purified by chromatography in system 2 in which it was eluted in HBV 3–5. Uridine diphosphate [1-^{14}C]-*N*-acetylglucosamine (5 μCi/μmole) was purchased from New England Nuclear, whereas inert UDPGNAc was obtained from Sigma Chemical Co. These migrated identically, 7 cm/hr toward the anode on flat-plate high-voltage

paper electrophoresis under 4000 V in pyridine acetate buffer, pH 3.5 (Wagner and Cynkin, 1968). Phenolsulfatase (Mylase P) was purchased from Wallerstein Co., Morton Grove, Ill.; β-*N*-acetylhexosaminidase, extracted from jack bean meal (Li and Li, 1970), was generously supplied by Dr. Yu-Teh Li. It had been purified as described (Li and Li, 1968), except that isoelectric focusing was replaced by gel filtration on Sephadex G-200, DEAE Sephadex A-50, and CM-Sephadex C-50.

Tissues. Human kidneys were obtained at nephrectomy for tumors in elderly males. The uninvolved areas were dissected and frozen until used. Human kidneys and livers without gross pathology were obtained from cadavers 2–36 hr post mortem. Fetal kidneys and livers were obtained at abortions and frozen promptly until use, except in 2 experiments in which the tissues were processed immediately.

Incubations. 15α-OHE$_1$. Tissue homogenates were prepared in ice in 4 parts (wet wt/volume) of 0.1 M sodium phosphate buffer, 0.05 M with respect to KCl, pH 7.4, in an all-glass Potter-Elvehjem homogenizer. In a typical run, 20,000 cpm of 15α-OHE$_1$ was incubated with 50 mg of tissue homogenate in 3.5 ml of the same buffer with shaking at 37° in the presence and absence of 1.4 mg of UDPGNAc. After 2 hr the homogenate was extracted 3 times with ether. In one study the kidney homogenate was maintained at 80° for 20 min prior to incubation. Preliminary evidence for the existence of *N*-acetylglucosaminyl transferase activity was indicated by a significantly greater amount of radioactivity in the aqueous phase of the incubation medium containing UDPGNAc. To characterize the product the aqueous phase was centrifuged and the supernatant was flash evaporated. The residue was chromatographed on Sephadex (Jirku and Levitz, 1969). Additional characterization of the *N*-acetylglucosaminide was obtained from chromatography in system 8 (HBV 2) and 9 (HBV 2-3). The radioactive material eluted from Sephadex between 36 and 60 ml was incubated in 0.2 ml of 0.05 M citrate buffer, pH 5.0, in the presence of 8 units of β-*N*-acetylhexosaminidase for 2 days at room temperature. A control was always run in which enzyme was not added. The hydrolysate was extracted with ether and the purity of the product was determined by the double isotope dilution method. [^{14}C]15α-OHE$_1$ was added to the ether extract and the mixture was chromatographed in system 1. The material eluted in HBV 11 was reduced with $NaBH_4$ and the product was chromatographed in system 3. At each step the ^3H:^{14}C ratio was determined.

15α-OHE$_1$-3 Sulfate. The conditions for incubation were as described in the previous section except that 15α-OHE$_1$-3 sulfate was substituted for 15α-OHE$_1$. The reaction was stopped by the addition of 4 parts of ethanol. The precipitate was removed, the supernatant was flash evaporated, and the residue was chromatographed on a 2 × 30 cm alumina column (Emerman *et al.*, 1967). The percentage of radioactivity eluted with 70–50% ethanol served to estimate transferase activity. To characterize the product it was first purified by chromatography in system 5 (HBV 3–4) or 6 (HBV 4). It proved necessary to remove TEAMS (originating from the partition system) prior to enzyme incubation. To do so, the sample was placed on a 1 × 50 cm Amerlite XAD-2 column, washed, and eluted essentially as described for urine samples (Bradlow, 1968). Then it was treated with β-*N*-acetylhexosaminidase. The solution was deproteinized by the addition of ethanol and the supernatant was chromatographed sequentially on alumina (90–80% ethanol) and in system 4 (HBV 5-6). The product was treated with 20 mg of phenolsulfatase in 5 ml of 0.1 M acetate buffer, pH 6.0, for 24 hr at 37°. Following extraction with ether, [^{14}C]15α-OHE$_1$ was added and the purity of the tritiated product was determined by the double isotope dilution method described in the previous section.

On the Position of Attachment of N-Acetylglucosamine. About 23,000 cpm of [^3H]15α-OHE$_1$GNAc in 0.2 ml of methanol was treated with 1.0 mg of $NaBH_4$ at 0° for 60 min. One drop of glacial acetic acid and 5 ml of water were added. The solution was extracted with ether and the aqueous phase was flash evaporated. The residue was dissolved in 1 ml of methanol and treated with excess diazomethane in 10 ml of methylene dichloride for 16 hr at 4°. Examination of an aliquot indicated incomplete methylation. Consequently, the treatment with diazomethane was repeated. The methylene dichloride was extracted with 10 ml of water. Hydrochloric acid (0.7 ml) was added to the aqueous phase and the solution was boiled for 40 min. The hydrolysate was cooled and extracted 3 times with 10 ml of ether. The combined ether fractions were washed once with water and then extracted with three 1-ml portions of 1 N NaOH. The NaOH was backwashed with ether. All extracts were assayed for radioactivity. [^{14}C]15α-OHE$_2$-3 methyl ether (4 mg) was added to the ether phase. The solution was evaporated and chromatographed in system 1 (HBV 9) and system 7 (HBV 4). The mixture was crystallized 3 times from methanol. The final crystals were treated with acetic anhydride in pyridine overnight and the resulting acetate was crystallized twice from ethanol. The ^3H:^{14}C ratios of the crystals and mother liquors were determined at each stage of purification.

Control Studies with Liver Homogenates. Since attempts to achieve the biosynthesis of *N*-acetylglucosaminides in liver homogenates failed, the following studies were performed with liver obtained from a 442-g fetus. First, the stability of the substrate to the reaction conditions was examined. [^3H]-15α-OHE$_1$ (20,000 cpm) was incubated with 50 mg of liver homogenate and 1.4 mg of UDPGNAc in 3.5 ml of buffer for 24 hr at 37°. The incubate was extracted with ether and the ether extract was analyzed for [^3H]15α-OHE$_1$ by the double isotope dilution method. Secondly, the liver was tested for *N*-acetylglucosaminidase activity. About 5000 cpm of [^3H]15α-OHE$_1$GNAc was incubated with and without 50 mg of liver homogenate in 3.5 ml of buffer for 24 hr. The incubates were extracted with ether and the ether phases were counted. Finally, the stability of UDPGNAc was assessed. Mixtures of 1.4 mg (1×10^5 cpm) of [^{14}C]UDPGNAc and 50 mg of homogenate in 3.5 ml of buffer were incubated for 0, 30, and 120 min at 37°. Four volumes of ethanol were added and the resulting supernatant was submitted to high-voltage paper electrophoresis at 4000 V in pH 3.5 pyridine acetate buffer for 3 hr. The paper was dried and scanned for radioactivity.

Determination of Radioactivity. Samples were counted in a Model 3375 Packard scintillation spectrometer essentially as described previously (Emerman *et al.*, 1967). The scintillant reported by Herberg (1960) was modified to contain the following components: 24 g of butyl-PBD (Nuclear Associates, Inc., Westbury, N. Y.), 240 g of naphthalene, 1200 ml of toluene, 1200 ml of dioxane, and 600 ml of methanol.

Results

Incubations with Kidney. Homogenates derived from adult kidneys obtained at nephrectomy and fetal kidneys were in-

TABLE II: Conversion of [^3H]15α-OHE$_1$ and [^3H]15α-OHE$_1$-3 Sulfate to *N*-acetylglucosaminides by Human Kidney Homogenates Fortified with UDPGNAc.

Expt No.	Kidney Type[a]	Substrate	Per Cent Conversion[b] + UDPGNAc	Per Cent Conversion[b] − UDPGNAc
1	Adult	[^3H]15α-OHE$_1$	56[c]	2.5
2	Fetus, 17 weeks	[^3H]15α-OHE$_1$	25[d]	5.8
3	Fetus, 19 weeks	[^3H]15α-OHE$_1$	64[d]	2.4
4	Adult	[^3H]15α-OHE$_1$-3S	85	
5	Adult	[^3H]15α-OHE$_1$-3S	65	2.7
6	Fetus, 17 weeks	[^3H]15α-OHE$_1$-3S	40	0.0
7	Fetus, 19 weeks	[^3H]15α-OHE$_1$-3S	16	3.2

[a] All specimens were frozen until used. The estimated gestation age for fetal kidneys is given. Adult kidneys were obtained at nephrectomy for tumor. The homogenates were incubated as described in the text. [b] Values indicated the per cent conversion into water-soluble product on ether–water partition when [^3H]15α-OHE$_1$ was the substrate and per cent conversion into product with the elution characteristic of 15α-OHE$_1$SGNAc on alumina, when [^3H]15α-OHE$_1$-3 sulfate was the substrate. Experiments were run in the presence (+) and absence (−) of UDPGNAc. [c] A homogenate maintained at 80° for 20 min prior to incubation gave 0.0 per cent conversion into GNAc. [d] Liver homogenates from these fetuses gave 1.7 and <6% conversions into water-soluble radioactivity. However, the values for the controls were the same.

cubated with [^3H]15α-OHE$_1$ in the presence and absence of UDPGNAc. The mixture was partitioned between ether and water. The results shown in Table II indicate that significant radioactivity was found in the aqueous phase only in incubations containing UDPGNAc. Similar incubations were conducted with [^3H]15α-OHE$_1$-3 sulfate as a substrate. Radioactive material with chromatographic properties identical with 15α-OHE$_1$SGNAc was dependent on the addition of UDPGNAc to the incubation medium (Table II).

Structure of the Products. These results suggested that [^3H]-15α-OHE$_1$ and [^3H]15α-OHE$_1$-3 sulfate were converted into *N*-acetylglucosaminide and sulfo-*N*-acetylglucosaminide, respectively. In representative incubations of adult and fetal kidneys the nature of the products was investigated further. In studies 1 and 2 (Table II), the radioactive material in the aqueous phase following incubation of kidney homogenate with [^3H]15α-OHE$_1$ and UDPGNAc was chromatographed on Sephadex where more than 90% of the activity was eluted in a

TABLE III: Double Isotope Dilution Data on 3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one.[a]

Expt No.	Crude	Chromatography System 1	Chromatography System 3[b]
1	1.53	1.56	1.56
2	4.44	4.38	4.48
4	4.28	4.29	4.22
6	3.42	3.46	3.43

[a] Following enzyme hydrolysis of each GNAc and SGNAc synthesized in the experiments listed in Table II (see text for details), at least 3800 cpm of [^{14}C]15α-OHE$_1$ was added to the tritiated ether extract. The mixture was submitted sequentially to the steps shown. See Table I for chromatography systems. Values are ^{3}H:^{14}C ratios. [b] The mixture was reduced with $NaBH_4$ prior to chromatography in this system.

TABLE IV: Double Isotope Dilution Data on 3-Methoxyestra-1,3,5(10)-triene-15α,17β-diol[a]

Purification steps		^3H:^{14}C Crystals	^3H:^{14}C Mother Liquors
Crude		1.17	
Chromatography, system 1		0.94	
Chromatography, system 7		0.89	
Crystallizations, methanol	1	0.91	1.14
	2	0.86	0.93
	3	0.84	0.89
Derivative[b]			
Crystallizations, ethanol	1	0.93	0.88
	2	0.85	0.87

[a] [^{14}C]3-Methoxyestra-1,3,5(10)-triene-15α,17β-diol (4 mg, 6500 cpm) was added to the radioactive material (5600 cpm) obtained following methylation of [^3H]15α-OHE$_2$GNAc, acid hydrolysis, and ether extraction. [b] Diacetate of 3-methoxyestra-1,3,5(10)-triene-15α,17β-diol.

TABLE V: Conjugation of [^3H]15α-OHE$_1$ by Fresh Fetal Kidney and Liver Homogenates Fortified with UDPGNAc.[a]

		Fetus 1 (14 Weeks)			Fetus 2 (17 Weeks)		
			% of Conjugate as			% of Conjugate as	
Tissue	UDPGNAc	% Conjugated[b]	GNAc[c]	Sulfate	% Conjugated[b]	GNAc[c]	Sulfate
Kidney	+	78	77	14	71	77	13
	−	38	45	42	22	33	62
Liver	+	17	2.6	89	7.9	0	88
	−	14			7.2		
Kidney plus	+	50			47		
Liver	−	33			23		

[a] Homogenates (50 mg) of kidney and/or liver in 3.5 ml of buffer were incubated for 2 hr with 20,000 cpm of [^3H]15α-OHE$_1$ in the presence (+) and absence (−) of exogenous UDPGNAc. [b] Per cent of ^{3}H in the water fraction following partition of incubate between ether and water. [c] Per cent of water fraction behaving like *N*-acetylglucosaminide (R_F 0.26) or sulfate (R_F 0.18) on thin-layer chromatography in $CHCl_3$–ethanol (7:4, v/v).

single symmetrical zone (36–60 ml). Treatment with β-*N*-acetylhexosaminidase rendered in each case 90% of the radioactivity ether soluble as compared to 3% for the control. Following admixture with [^{14}C]15α-OHE$_1$ the hydrolysate was submitted to the purification steps shown in Table III. It is evident that there is no change in the ^{3}H:^{14}C ratios.

The products of incubation of [^3H]15α-OHE$_1$-3 sulfate with kidney homogenates were investigated in experiments 4 and 6 (Table II). With adult kidney homogenate, the material eluted with 70–50% ethanol from alumina was chromatographed in system 5. About 75% of the radioactivity was eluted in HBV 3–4. With fetal kidney as the tissue source the material from alumina yielded a single symmetrical zone (HBV 4) in system 6. In each experiment the product was treated with β-*N*-acetylhexosaminidase and the hydrolysate was chromatographed sequentially on alumina and in system 4. In each instance an average of over 90% of the radioactivity corresponded to 15α-OHE$_1$-3 sulfate, as compared with 2% in control incubations. Subsequent treatment with phenolsulfatase rendered 94% of the radioactivity ether soluble, whereas in the controls the values were less than 2%. The ether-soluble material was mixed with [^{14}C]15α-OHE$_1$ and purified according to the steps shown in Table III. It is evident that the ^{3}H:^{14}C ratios remained constant.

The position of attachment of *N*-acetylglucosamine to 15α-OHE$_1$ was examined indirectly. The 15α-OHE$_1$GNAc synthesized by adult kidney was reduced to 15α-OHE$_2$GNAc by $NaBH_4$. The yield was 82%. Following sequential treatment with diazomethane, acid hydrolysis and partition between ether and 1 N NaOH, about 33% of the original radioactivity was found in the final ether extract. [^{14}C]15α-OHE$_2$-3 methyl ether (4 mg) was added and the mixture was analyzed for purity by the method of double isotope dilution. The data in Table IV indicate that there was a 24% decrease in the ^{3}H:^{14}C ratios during the initial purification steps and then constancy was achieved. The implication is that *N*-acetylglucosamine is attached at position 15 in 15α-OHE$_1$GNAc.

On the N-Acetylglucosaminyl Transferase Activity in Adult and Fetal Liver. Repeated attempts to demonstrate *N*-acetylglucosaminyl transferase activity in liver were unsuccessful. Thus, in experiments 2 and 3 (Table II) liver homogenates were incubated in parallel with the kidney specimens. About 5% of the tritium was rendered ether soluble, an amount equal to that obtained for the controls. Similarly, livers obtained from cadavers 3- to 36-hr post mortem also gave negative results. On the other hand, a 24-hr post-mortem kidney specimen gave a 25% conversion into water-soluble radioactive material. The control was 5%.

The possibility that an extremely labile transferase was present in fetal liver was investigated. Fetal kidney and liver were obtained immediately after hysterotomy and incubated in the usual fashion. The results shown in Table V are similar to those obtained with frozen specimens (Table II) except that in the absence of exogenous UDPGNAc appreciable radioactivity was found in the aqueous fraction. There is no evidence for the synthesis of *N*-acetylglucosaminides by liver homogenates. Virtually all the water-soluble radioactive material behaved like sulfate on thin-layer chromatography (chloroform–ethanol, 7:4 v/v). On the other hand, with fresh kidney *N*-acetylglucosaminide synthesis was stimulated by the addition of UDPGNAc. Even without the addition of UDPGNAc some *N*-acetylglucosaminide synthesis appears to have occurred. The addition of liver homogenate depressed but did not abolish the capability of the kidney homogenate to conjugate [^3H]15α-OHE$_1$. The nature of the products formed in this experiment was not investigated.

The absence of transferase activity in the fetal liver required conducting control experiments centering on the stability of [^3H]15α-OHE$_1$, [^3H]15α-OHE$_1$GNAc, and [^{14}C]UDPGNAc under the incubation conditions. Following 24-hr incubation of [^3H]15α-OHE$_1$ in fetal liver homogenate fortified with UDPGNAc the medium was extracted with ether. About 97% of the radioactivity was ether soluble and double isotope dilution analysis indicated that 90% of this material was unchanged [^3H]15α-OHE$_1$. In a parallel flask [^3H]15α-OHE$_1$-GNAc was incubated with fetal liver under the same conditions for 24 hr. Ether extraction removed less than 3% of the radioactivity, a value similar to that obtained in a control containing no homogenate. Finally, the stability of UDPGNAc was tested. Samples of [^{14}C]UDPGNAc (1.4 mg) were in-

cubated with 50 mg of fetal liver homogenates for 0, 30, and 120 min. Four parts of ethanol was added and the supernatants were analyzed for [^{14}C]UDPGNAc by high-voltage paper electrophoresis. A peak with about one-half the mobility of UDPGNAc developed in the 30- and 120-min incubations. However, after 120 min 46% of the ^{14}C incubated still had the mobility of UDPGNAc. Nevertheless, a further control was run in which additions of UDPGNAc (1.4 mg) were made at 30 and 60 min during the course of a 2-hr incubation which contained [^{3}H]15α-OHE$_1$, 1.4 mg of UDPGNAc, and 50 mg of fetal liver homogenate at the start. Less than 2% of the radioactivity was rendered water soluble.

Discussion

This report presents proof that the human adult and fetal kidney contain an *N*-acetylglucosaminyl transferase which utilizes either 15α-OHE$_1$ or 15α-OHE$_1$-3 sulfate as substrate and that the products are the respective β-*N*-acetylglucosaminides. The evidence is as follows. The homogenates required fortification with UDPGNAc for significant product formation. The product was hydrolyzed by a pure preparation of β-*N*-acetylhexosaminidase, which does not hydrolyze α-glycoside linkages (Li and Li, 1970). Finally, following hydrolysis the steroid was identified as 15α-OHE$_1$ by the reliable method of double isotope dilution.

The position of attachment of *N*-acetylglucosamine to 15α-OHE$_1$ was determined indirectly. The compound was reduced with $NaBH_4$ to 15α-OHE$_2$GNAc which was treated with diazomethane under conditions in which phenols but not neutral hydroxyls are methylated. Finally, acid hydrolysis produced the 3-methyl ether of 15α-OHE$_2$. Thus barring the unlikelihood of an enol *N*-acetylglucosaminide at C-17 (the relatively high stability of 15α-OHE$_1$GNAc to acid and base at room temperature militate against this), the conjugate is identified as 3-hydroxy-17-oxoestra-1,3,5(10)-trien-15α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside. By analogy, and because only one position is available for conjugation, the product of reaction of 15α-OHE$_1$-3 sulfate is the corresponding 15-*N*-acetylglucosaminide. Unequivocal assignments of these structures must await comparisons with authentic crystalline compounds.

Perhaps a surprising aspect of this study was the failure to detect *N*-acetylglucosaminyl transferase activity in liver. Human liver is rich in glucuronyl transferase (Slaunwhite *et al.*, 1964) and sulfotransferase activities (Dao and Libby, 1968). Furthermore, a detailed study of a subject who received labeled estrone sulfate intravenously revealed that [^{3}H]15α-OHE$_1$SGNAc and [^{3}H]15α-OHE$_2$SGNAc are major biliary metabolites (Jirku and Levitz, 1969). Fresh adult liver has not been available for *in vitro* experiments and it has not been feasible to perform critical *in vivo* experiments. Thus there are 2 alternatives. *N*-Acetylglucosaminides may be synthesized in kidney and elsewhere and then transported to the liver for secretion in the bile. On the other hand, it must be considered that the negative result with liver homogenate may not reflect the situation *in vivo*.

Although the existence of a labile *N*-acetylglucosaminyl transferase in adult human liver is still under consideration, it does not appear likely that such activity exists in fetal liver up to midgestation. Fresh fetal liver homogenates failed to biosynthesize *N*-acetylglucosaminides. It would appear that the absence of activity from fetal liver cannot be attributed solely to inhibition. Experiments in which mixtures of fetal liver and kidney homogenates were incubated with [^{3}H]15α-OHE$_1$ and UDPGNAc indicate that liver has an inhibitory effect, but does not abolish the transferase activity of kidney, when equal amounts of both tissues are used. The nature of the inhibition was not investigated. Also it has been ruled out that negative results with fetal liver homogenates can be attributed to instability of substrate, product, or UDPGNAc in the system.

The organ localization of *N*-acetylglucosaminyl transferase activity has been studied systematically in the rabbit with 17α-estradiol 3-glucuronide as the substrate (Collins *et al.*, 1968). Activity was greatest in the liver, whereas kidney and large intestine displayed, respectively, 0.4 and 0.2% as much activity. The endocrine glands and reproductive organs were devoid of activity. The only other steroid *N*-acetylglucosaminide reported is 3β-sulfato-5-pregnen-20α-yl 2′-acetamido-2′-deoxy-β-D-glucopyranoside (Arcos and Lieberman, 1967; Matsui and Fukushima, 1969). However, the conjugate was isolated from human urine, so that the organ of synthesis cannot be pinpointed.

The substrate specificity of *N*-acetylglucosaminyl transferase merits serious attention. Studying a limited number of steroids, Layne and his associates concluded that in the rabbit three conditions needed to be satisfied for the transfer of *N*-acetylglucosamine to occur. First, only estrogens participate in the reaction. Secondly, prior conjugation at C-3 with either glucosiduronate or sulfate is required (Collins *et al.*, 1968, Collins and Layne, 1969). Thirdly, the 17α-hydroxy group is the preferred recipient of *N*-acetylglucosamine (Collins and Layne, 1968). Similar systematic studies have not been conducted with enzyme systems from human sources. However, careful examination of human bile and urine for estrogen conjugates suggests that *N*-acetylglucosaminidation may be specific for 15α-hydroxyestrogens (Jirku and Levitz, 1969, 1970). In addition to the extensive studies on 15α-OHE$_1$GNAc, 15α-OHE$_1$SGNAc, 15α-OHE$_2$GNAc, and 15α-OHE$_2$SGNAc, preliminary evidence has been obtained for the excretion of an *N*-acetylglucosaminide and a sulfo-*N*-acetylglucosaminide of estra-1,3,5(10)-triene-3,15α,16α,17β-tetrol in the urine following the administration of tritiated tetrol to a woman (H. Jirku and M. Levitz, unpublished observation). In closing it is interesting to note that, in contrast to the rabbit, conjugation at C-3 is not a prerequisite for estrogen *N*-acetylglucosaminyl transferase activity in the human.

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Addendum

Since the submission of this manuscript we investigated the normal part of fresh and freshly frozen human liver which had been dissected from a carcinomatous area following partial hepatectomy. Utilizing [^{3}H]15α-OHE$_1$ and [^{3}H]15α-OHE$_1$-3S

as substrates in 20% homogenate fortified with UDPGNAc, tritiated products with chromatographic properties of 15α-OHE_1GNAc and 15α-OHE_1SGNAc, respectively, were obtained. The 15α-OHE_1GNAc was chromatographed on alumina and system 9 (Table I), whereas the 15α-OHE_1SGNAc was chromatographed on alumina and system 5. The yields were comparable to the highest reported for kidney in Table II, but it is pointed out that higher concentrations of liver homogenates were used.

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