[Chem. Pharm. Bull. 20(12)2607—2612(1972)]

UDC 547.92.057 : 542.98

Studies on Steroid Conjugates. XI. Biosynthesis of 16-Epiestriol Glucosiduronate[1]

Toshio Nambara, Yasuhiko Matsuki, and Motoko Kurata

Pharmaceutical Institute, Tohoku University[2]

(Received May 8, 1972)

Biosynthesis of 16-epiestriol glucosiduronate was attempted with liver and small intestine preparations from several different species. Incubation of ^{3}H-16-epiestriol with microsomal or supernatant fractions in the presence of UDPGA yielded the glucosiduronates whose separation was readily attained by column chromatography on Amberlite XAD-2 resin. The structure of 16-epiestriol glucosiduronates was elucidated by direct comparison with the synthetic samples on the thin-layer chromatogram, leading to the acetate-methyl ester for the reverse isotope dilution method and hydrolytic cleavage with β-glucuronidase. There could be seen the distinct difference in the position of conjugation depending upon the species and organ as listed in Table IV and V. The specificity and multiplicity of UDP-glucuronyltransferase toward 16-epiestriol as an acceptor have been discussed.

It is sufficiently substantiated that in the steroid glucosiduronate formation the glucuronic acid moiety is transferred from uridine-5′-diphosphoglucuronic acid (UDPGA) to the steroid under the influence of UDP-glucuronyltransferase (EC 2.4.1.17).[3] Recently considerable attentions have been drawn to the metabolic role of conjugation with the steroid hormones.[4] On the other hand, the physiological significance of 16-epiestriol besides the classical estrogen in the feto-placental unit seems to be an attractive problem. In the previous studies of this series three possible 16-epiestriol monoglucosiduronates have been synthesized from necessity of the authentic samples.[5] The variation of glucuronyltransferase activity in anyone species toward the different substrates was reported.[6] It was also suggested that one common active site may be involved in the enzymatic glucuronylation of several compounds.[7] A particular interest in these respects prompted us to explore the specificity and multiplicity of 16-epiestriol glucuronyltransferase in liver and small intestine of several species. This paper deals with the biosynthesis and proof of structure of 16-epiestriol glucosiduronates formed by the UDP-glucuronyltransferase system.

1) Part X: T. Nambara, S. Honma, and K. Kanayama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2235 (1972). This paper also constitutes Part LX of the series entitled "Analytical Chemical Studies on Steroids"; Part LIX: T. Nambara, S. Honma, and K. Kanayama, *Chem. Pharm. Bull.* (Tokyo), **20**, 2235 (1972). Following trivial names are used: 16-epiestriol, estra-1,3,5(10)-triene-3,16β,17β-triol; estriol, estra-1,3,5(10)-triene-3,16α,17β-triol; estradiol, estra-1,3,5(10)-triene-3,17β-diol.

2) Location: *Aobayama. Sendai.*

3) K.J. Isselbacher, "Recent Progress in Hormone Research," Vol. 12, ed. by G. Pincus, Academic Press, New York, 1956, pp. 134—151.

4) H.E. Hadd and R.T. Blickenstaff, "Conjugates of Steroid Hormones," Academic Press, New York, 1969, pp. 293—318; D.S. Layne, "Metabolic Conjugation and Metabolic Hydrolysis," Vol. 1, ed. by W.H. Fishman, Academic Press, New York, 1970, pp. 21—52.

5) T. Nambara, Y. Matsuki, and T. Chiba, *Chem. Pharm. Bull.* (Tokyo), **17**, 1636 (1969); T. Nambara, Y. Matsuki, and Y. Kawarada, *ibid.*, **19**, 844 (1971); T. Nambara, Y. Matsuki, and N. Miyazaki, *ibid.*, **20**, 842 (1972).

6) I.D.E. Storey, *Biochem. J.*, **95**, 209 (1965); T.E. Gram, A.R. Hansen, and J.R. Fouts, *ibid.*, **106**, 587 (1968); A.R. Temple, M.S. Clement, and A.K. Done, *Proc. Soc. exp. Biol. Med.*, **128**, 307 (1968).

7) G.J. Mulder, *Biochem. J.*, **125**, 9 (1971).

Experimental

Materials——Generally labeled ^{3}H-16-epiestriol was obtained from Daiichi Pure Chemicals Co., Tokyo, and purified by column chromatography on Al_2O_3 prior to use to constant specific radioactivity (4.82 μCi/μmole). UDPGA Na salt (Sigma Chemical Co., St. Louis), beef-liver β-glucuronidase (EC 3.2.1.31) (Tokyo Zōkikagaku Co., Tokyo) and Amberlite XAD-2 resin (Rohm & Haas Co., Philadelphia) were purchased. D-Glucaro-1,4-lactone was kindly supplied by Tokyo Biochemical Research Institute. 16-Epiestriol and its triacetate, 16-epiestriol 3-, 16- and 17-monoglucosiduronates and their acetate-methyl esters were prepared in this laboratory.[5]

Animals——Hartley strain male guinea pigs (body weight 500—600 g), domestic strain white male rabbit (body weight 2.8 kg) and Wistar strain male rats (body weight 240—300 g) were used.

Enzyme Preparations——Liver and small intestine were obtained from decaptitated animals and washed with 0.25 M sucrose. Each 5 g piece, accurately weighed, was homogenized with 0.25M sucrose (40 ml), centrifuged at $10000\times g$ for 30 min and the supernatant was then centrifuged at $105000\times g$ for 60 min. The microsomal pellets were washed with 0.25M sucrose and suspended in 0.15M Sörensen phosphate buffer (pH 7.4) (5 ml). The supernatant was diluted with 0.15M phosphate buffer (pH 7.4) to make the total volume 80 ml. Sample of human liver, secured from a 37-year-old male at operation, was processed in the manner as described above. The preparations were stored at $-10°$ until used for the incubation experiment.

Incubation——Each incubation mixture contained the following: microsomal preparation (1 ml, equivalent to 1 g of tissue), ^{3}H-16-epiestriol (32 μg, 0.84 μCi), UDPGA (170 μg), D-glucaro-1,4-lactone (33 μg) and sufficient 0.15M phosphate buffer (pH 7.4) to make the total volume 2 ml. The supernatant preparation (16 ml, equivalent to 1 g of tissue) was similarly processed as described above to make the final volume 20 ml. These were incubated at 37° for 60 min in a shaker-incubator with air as the gas phase.

Separation of 16-Epiestriol Glucosiduronates——To the incubation mixture was added twofold volume of 95% EtOH and allowed to stand in a refrigerator overnight. After removal of the precipitate by centrifugation, the supernatant was concentrated *in vacuo* to *ca.* 2 ml below 45°. The residue was dissolved in 50% MeOH (2—3 ml), percolated through a column packed with Amberlite XAD-2 resin (30 ml) and then washed with distilled water (50 ml). The conjugated and free 16-epiestriol were eluted with 40% EtOH (100 ml) and 90% EtOH (100 ml), successively. Each effluent was evaporated to dryness *in vacuo* and the residue was redissolved in 50% MeOH (5 ml). An aliquot (0.5 ml) of this solution was assayed for radioactivity.

Counting of Radioactivity——Samples containing ^{3}H were counted in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer. For toluene-soluble samples a solution of 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (400 mg) in toluene (1 liter) was used as a scintillator. Aqueous samples were counted in Bray's scintillator, composed of naphthalene (60 g), 2,5-diphenyloxazole (4 g), 1,4-bis-[2-(5-phenyloxazolyl)]benzene (200 mg), MeOH (100 ml), ethylene glycol (20 ml) and sufficient dioxane to make the total volume 1 liter.[8]

Transformation of 16-Epiestriol 3-Glucosiduronate into Acetate-Methyl Ester——A portion of the glucosiduronate fraction was taken up and dissolved in MeOH (1 ml). To this solution was added an ethereal solution of CH_2N_2 and allowed to stand at room temperature for 1 hr. After addition of a drop of AcOH the solution was evaporated with an aid of N_2 gas stream. The residue was dissolved in pyridine (0.4 ml)–Ac_2O (0.2 ml) and allowed to stand at room temperature overnight. On usual work-up the product was diluted with an appropriate amount of 16-epiestriol 3-glucosiduronate acetate-methyl ester as a carrier and recrystallized repeatedly to the constant specific radioactivity.

Enzymatic Hydrolysis of 16-Epiestriol Glucosiduronates——To a solution of 16-epiestriol glucosiduronates in 0.1M acetate buffer (pH 4.7) (1.2 ml) was added beef-liver β-glucuronidase (2600 Fishman units) and the resulting solution was incubated at 37° for 48 hr. The incubated fluid was saturated with NaCl and then extracted with AcOEt (30 ml$\times$3). The organic layer was separated and evaporated *in vacuo*. The residue was dissolved in EtOH (1 ml) whose an aliquot (0.2 ml) was assayed for radioactivity. A portion of the hydrolyzate was submitted to thin-layer chromatography (TLC) employing benzene–ether (1: 2) and hexane–AcOEt (2:1) as developing solvent whereby 16-epiestriol was detected as a spot with *Rf* values of 0.54 and 0.22, respectively. At the same time hydrolysis with β-glucuronidase in the presence of D-glucaro-1,4-lactone (33 μg) was similarly carried out and the amount of 16-epiestriol liberated was determined.

Thin-Layer Chromatography (TLC)——The chromatogram was run on the plate (10$\times$40 cm) with a layer (0.25 mm) of Silica gel G (E. Merck AG, Darmstadt) activated at 120° for 2 hr. For 16-epiestriol monoglucosiduronates the system $CHCl_3$/iso-PrOH/HCOOH (15: 5: 4) was used after tank equilibration for 30 min. Mobility was expressed as *Rt* value, traveled distance from the origin for 18 hr.

Paper Chromatography (PPC)——Whatman No. 1 filter paper (3$\times$30 cm) was used for descending chromatography at 25°. The system (iso-Pr)$_2$O/*tert*-BuOH/NH_4OH/H_2O (4: 6: 3: 8) was used after tank equilibration for 24 hr. Mobility was expressed as *Rt* value obtained by development for 66 hr.

8) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).

Result and Discussion

First, examinations were made on the chromatographic separation of three possible 16-epiestriol monoglucosiduronates employing the synthetic specimens. The thin-layer and paper chromatographic data under the suitable conditions are listed in Table I. The

Table I. Thin-Layer and Paper Chromatographic Data of 16-Epiestriol Monoglucosiduronates

Compound	Distance traveled (Rt)	
	TLC[a]	PPC[b]
3-Glucosiduronate	9.8	9.8 cm
16-Glucosiduronate	11.4	15.1
17-Glucosiduronate	10.4	12.8

a) solvent: $CHCl_3$/iso-PrOH/HCOOH (15:5:4), adsorbent; Silica gel G (E. Merck AG, Darmstadt), developing time: 18 hr
b) solvent: $(iso\text{-}Pr)_2O$/*tert*-BuOH/NH_4OH/H_2O (4:6:3:8), filter paper: Whatman No. 1, developing time: 66 hr

Table II. Determination of Purity of ^{3}H-16-Epiestriol 3-Glucosiduronate Acetate-Methyl Ester by Reverse Isotope Dilution

No.	Crystallization from	Specific activity (dpm/mg)			
		Guinea pig		Rabbit	
		Crystals	Mother liquor	Crystals	Mother liquor
1	acetone–hexane	280	310	270	250
2	EtOH	280	310	260	260
3	EtOH	290	300	250	250

3-glucosiduronate was most polar of the three and could be easily differentiated from the others. The chromatographic behaviors of the 16- and 17-glucosiduronates were quite similar, but the former was slightly less polar than the latter. Accordingly, these two could be also distinguished with each other when developed for a prolonged period.

An efficient method for the separation of 16-epiestriol and its glucosiduronates from the biological fluid was then investigated. As illustrated in Fig. 1, the 3-glucosiduronate was separated from the unchanged aglycone with the relative ease by column chromatography on Amberlite XAD-2 resin.[9] The radioactivity of the glucosiduronate fraction eluted with 40% ethanol expressed the rate of conjugation. By this procedure ^{3}H-16-epiestriol added to the incubation mixture was recovered in the combined free and conjugate fractions at the rate of *ca.* 89%.

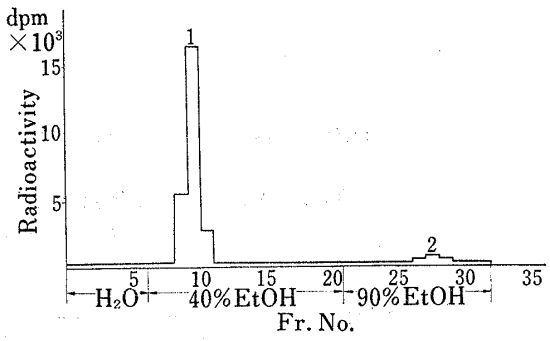

Fig. 1. Separation of 16-Epiestriol and Its 3-Glucosiduronate from Incubated Fluid with Rabbit Liver Preparation by Column Chromatography on Amberlite XAD-2 Resin

1: 16-epiestriol 3-glucosiduronate; 2: 16-epiestriol

9) H.L. Bradlow, *Steroids*, **11**, 265 (1968).

Structure elucidation of the steroid glucosiduronates was achieved by direct comparison with the synthetic samples and by hydrolytic cleavage with β-glucuronidase. A portion of the conjugate was submitted to TLC together with the reference compounds under the conditions established above. The 3-glucosiduronate was further transformed into the acetate–methyl ester by treatment with diazomethane and then with acetic anhydride-pyridine in the usual manner. The radiochemical homogeneity of this derivative could be determined by the reverse isotope dilution method. As listed in Table II the purity of the 3-glucosiduronate formed with guinea pig and rabbit liver microsomes was confirmed by this procedure. On treatment with acetic anhydride-pyridine 16-epiestriol liberated by enzymatic hydrolysis was led to the triacetate, which was identified by the reverse isotope dilution method. The results on a typical experiment with the 3-glucosiduronate are listed in Table III.

Table III. Determination of Purity of ^{3}H-16-Epiestriol Triacetate by Reverse Isotope Dilution

No.	Crystallization from	Specific activity (dpm/mg)	
		Crystals	Mother liquor
1	MeOH	5920	6380
2	MeOH	5720	5500
3	acetone–hexane	5370	5460

Table IV. Comparison of Ability to Conjugate 16-Epiestriol by Liver Preparations from Four Different Species

Species	Conjugation			Hydrolysis rate (%)	
	Fraction	Position	Rate (%)	Inh (−)	Inh (+)
Guinea pig	M	3	88.6	86.4	7.3
	S	3	6.2	84.2	14.3
Rabbit	M	3	87.7	105.1	12.8
	S	3	4.9	90.8	18.9
Rat	M	16	8.2	76.1	39.7
	S	16 or 17	7.2	87.0	37.4
Man	M	16	22.9	95.5	14.3
	S	16	8.8	89.6	15.4

M: microsomes; S: 105000 × *g* supernatant; Inh: inhibitor

Table V. Comparison of Ability to Conjugate 16-Epiestriol by Intestine Preparations fromThree Different Species

Species	Conjugation			Hydrolysis rate (%)	
	Fraction	Position	Rate (%)	Inh (−)	Inh (+)
Guinea pig	M	3	7.0	76.8	16.8
	S	17	4.3	78.7	12.9
Rabbit	M	17	9.2	85.3	38.5
	S	17	5.0	89.1	34.8
Rat	M	16	6.8	88.1	33.6
	S	16 or 17	3.8	94.6	41.5

M: microsomes; S: 105000 × *g* supernatant; Inh: inhibitor

The data on a comparison of the ability of liver preparations from four species to conjugate 16-epiestriol are collected in Table IV. The rates of enzymatic hydrolysis of the con-

jugate and of inhibition of β-glucuronidase by D-glucaro-1,4-lactone are also included. These results verified the formation of a β-glucosiduronate linkage. The relative ability of small intestine preparations from three species to form 16-epiestriol glucosiduronates and the hydrolysis rate of the conjugate with or without addition of the inhibitor are also tabulated (see Table V). The ability to conjugate was compared under the arbitrary conditions because the optimal conditions were not established for each species and organ.

It is well established that in the enzymatic synthesis of glucosiduronates the glucuronic acid moiety of UDPGA is transferred by the catalysis of transferase to an acceptor. The high substrate specificity of this enzyme has already been shown with a wide variety of the non-steroidal compounds.[10] The evidences presented in this paper demonstrated that liver and intestine of several species such as guinea pig, rabbit, rat, and man were capable of catalyzing transfer of glucuronic acid to 16-epiestriol. Furthermore, conjugation occurred at the three different hydroxyl groups in the molecule depending upon the species difference. The liver preparation of guinea pig and rabbit glucuronylated estriol and estradiol selectively at the C-3 hydroxyl group.[11] The formation of 16-epiestriol 3-glucosiduronate with liver microsomes of these two species further supports the greater specificity toward the phenolic than the alcoholic hydroxyl groups. Slaunwhite, *et al.* were able to glucuronylate estriol at C-16 employing the human liver preparation, whereby 16α-glucuronyltransferase appeared to be a unique feature of human liver from the competitive inhibition experiments.[12] The present study revealed that glucuronic acid was transferred to the 16β-hydroxyl group of 16-epiestriol by human liver. It is of particular interest that two separate enzymes with the stereospecificity for the epimeric hydroxyl groups reside in the same cellular fraction. Breuer and his co-workers reported the occurrence of an enzyme conjugating the 16α-hydroxyl group of estriol with glucuronic acid in the cytosol fraction as well as microsomes of human liver.[12,13] These findings are in accord with our results that both fractions of rabbit, guinea pig and human liver mediated the enzymatic synthesis of the same 16-epiestriol glucosiduronate, respectively. In the case of the rat, however, conjugation was so poor that insufficient product was obtained for the definite determination of the attached position in ring D.

The UDP-glucuronyltransferase has been found in several species in a number of organs, including liver, kidney, stomach and intestine. It has recently been clarified that the soluble UDP-glucuronyltransferase which catalyzes the formation of estriol 17-glucosiduronate is localized in the ground plasma (150000×***g*** supernatant) of human intestine.[14] In the rabbit the properties of this enzyme appear to be characteristic to the organ in such a manner that liver and intestine effected transfer of glucuronic acid to the different positions of 16-epiestriol, C-3, and C-17 respectively. The divergence of glucuronylation can be also deduced from the results with the microsomal and supernatant fractions of guinea pig intestine.

The present findings on the specificity of conjugation depending upon the species and organ will be helpful for the enzymatic preparation of the isotope-labeled 16-epiestriol glucosiduronates in the biochemical studies. The feature of hepatic UDP-glucuronyltransferase seems to reflect on the nature of the urinary conjugate and hence 16-epiestriol is excreted as the 16-glucosiduronate in the human pregnancy urine.[15] However, it remains to be investigated whether or not the specificity and multiplicity of UDP-glucronyltransferase possess any

10) G.J. Dutton, "Glucuronic Acid, Free and Combined," ed. by G.J. Dutton, Academic Press, New York, 1966, pp. 185—299.
11) U. Goebelsmann, E. Diczfalusy, J. Katz, and M. Levitz, *Steroids*, **6**, 859 (1965); H. Jirku and D.S. Layne, *Biochemistry*, **4**, 2126 (1965); Y.A. Sa'at and W.R. Slaunwhite, Jr., *Steroids*, **13**, 545 (1969).
12) W.R. Slaunwhite, Jr., M.A. Lichtman, and A.A. Sandberg, *J. Clin. Endocrinol. Metab.*, **24**, 638 (1964).
13) G.S. Rao, M.L. Rao, and H. Breuer, *Biochem. J.*, **119**, 635 (1970).
14) K. Dahm and H. Breuer, *Biochim. Biophys. Acta*, **113**, 404 (1966); *idem*, *ibid.*, **128**, 306 (1966); G.S. Rao, M.L. Rao, and H. Breuer, *Biochem. J.*, **118**, 625 (1970).
15) T. Nambara, Y. Matsuki, J. Igarashi, and Y. Kawarada, to be published.

physiological significance in the metabolism of the female hormone. It is hoped that further studies in progress in this laboratory will provide the more precise knowledge on these interesting problems.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, which is gratefully acknowledged.