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Pathway and Stereochemistry of the Formation of Estriols in Man*

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ABSTRACT: Following the administration of estradiol-16β-^{3}H and -16α-^{3}H to human subjects the tritium content of the urinary estriol and estra-1,3,5(10)-triene-3,16β,17β-triol (16-epiestriol) was determined. From the values it is evident that the hydroxylation at C-16 whether α or β proceeds by replacement of hydrogen and that no common enol form is involved in the reaction. Further, 16-keto compounds cannot be considered as intermediates in the biosynthesis of estriol or 16-epiestriol. The difference in the recovery of tritium in urine and body water in the 16β-^{3}H and 16α-^{3}H studies suggests that the substantial portion of the administered dose which is never recovered in the urine is altered by 16α and not 16β substitution.

The metabolism of the female sex hormone in man is distinguished by competitive hydroxylation at C-2 and C-16 (Fishman *et al.*, 1965). The latter course, formation of estriol and its 16β-epimer, is sometimes quantitatively the more important and the metabolic sequence leading to these products is, therefore, of considerable interest. It has been shown (Fishman *et al.*, 1960) that oxidation of estradiol to estrone is a requisite stage of the *in vivo* formation of estriol and 16-epiestriol[1] and that 16-hydroxylation of estradiol, as such, does not occur to any significant extent (Fishman *et al.*, 1961). The subsequent steps by which estriol and 16-epiestriol are derived have been the subject of considerable speculation, in particular with respect to the 16-keto compounds, 16-ketoestradiol (Layne and Marrian, 1958) and 16-ketoestrone (Serchi, 1953; Slaunwhite and Sandberg, 1956). It has been suggested that these compounds are either precursors for estriol and 16-epiestriol or oxidation products of the estriols in the human (Breuer, 1960; Dorfman and Ungar, 1965) and both *in vivo* (Levitz *et al.*, 1956, 1958, 1960; Stimmel, 1958; Nocke *et al.*, 1961) and *in vitro* (Breuer and Knuppen, 1958; Breuer *et al.*, 1958, 1959); evidence has been offered for either point of view.

The stereochemical nature of C-16 hydroxylation leading to estriol and 16-epiestriol is of particular interest. This question arises because estrone is the substrate for C-16 hydroxylation, and therefore an enolizable hydrogen α to a ketone is involved. It has been demonstrated (Bergstrom *et al.*, 1958; Corey *et al.*, 1958; Hayano *et al.*, 1958) that enzymatic hydroxylation at other unactivated (in the chemical sense) positions in the steroid molecule proceeds with replacement rather than displacement of the hydrogen involved. This stereochemistry need not apply to hy-

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[1] Abbreviations used: 16-epiestriol, estra-1,3,5(10)-triene-3,16β,17β-triol; 16α-hydroxyestrone, 3,16α-dihydroxyestra-1,3,-5(10)-trien-17-one; 16β-hydroxyestrone, 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one; epiestriol acetonide, 16β,17β-isopropylenedioxyestra-1,3,5(10)-trien-3-ol; 16-ketoestradiol, 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one; 16-ketoestrone, 3-hydroxyestra-1,3,5(10)-triene-16,17-dione; 2-methoxyestrone, 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one.

droxylation at carbons where an enol or some other form of participation of the π electrons of the adjacent unsaturation may be involved. A study of the stereochemistry of hydroxylation of the enolizable C-6 in the Δ^4-3-keto steroids (Baba *et al.*, 1963) was inconclusive since only one stereospecifically labeled epimer substrate was used and only one epimeric C-6 hydroxylated product was obtained. The stereochemistry of the hydroxylation leading from estrone to estriol and 16-epiestriol is thus of general interest since, in all likelihood, it would also apply to other enzymatic hydroxylations α to a carbonyl group.

The design of the experiment which would provide answers to the above questions required the administration of estradiol labeled stereoselectively with tritium at the 16α and 16β positions. The necessary compounds became available following the successful preparation of the corresponding deuterio derivatives (Fishman, 1965) where the orientation of the isotope was demonstrated by nuclear magnetic resonance spectrometry.

Material and Methods

Subjects. Subject SO was a man 42 years of age who had had a myocardial infarction 3 years earlier but was well at the time of study. His weight was 84 kg, creatinine 2.0 g/24 hr, lean body mass 64 kg, calculated body water 46.5 l. (Miller and Blyth, 1952). Subject BY was a normal woman, 32 years of age, weight 55 kg, creatinine 1.1 g/24 hr, lean body mass 44.7 kg, calculated body water 32.6 l. Subject MR was a woman 63 years of age with osteoporosis, weight 53 kg, creatinine 0.9 g/24 hr, lean body mass 40.3 kg, calculated body water 29.4 l. All three subjects were free of liver, kidney, and endocrine dysfunction.

17β-Estradiol-4-^{14}C (31.8 mc/mmole) was obtained commercially and was at least 97% radiochemically pure by isotope dilution analysis. Estradiol-16β-^{3}H (3.2×10^6 cpm/mg) and estradiol-16α-^{3}H (9.7×10^6 cpm/mg) were synthesized in this laboratory (Fishman, 1965, 1966) and were of better than 95% purity, as determined by isotope dilution analysis. The location of the isotope in each compound was shown to be at least 99% at C-16, since <0.5% of the radioactivity remained after equilibrating the corresponding 17-keto compounds in alkali. From the evidence of the corresponding deuterium compounds prepared by identical procedures (Fishman, 1965) the 16β-^{3}H-estradiol contained <10% of the 16α-^{3}H isomer, while the 16α-^{3}H-estradiol contained 15–20% of the isomeric 16β-^{3}H-labeled material. The inert compounds, estrone, estradiol, 2-methoxyestrone, estriol, and 16-epiestriol were obtained commercially. Thin layer chromatography and melting point determination established their purity. Solvents were redistilled prior to use.

Before administration, the mixture of estradiol-4-^{14}C and estradiol-16β-^{3}H or -16α-^{3}H was dissolved in 2 ml of redistilled, pyrogen-free propylene glycol. An aliquot was retained to serve as standard and the remainder was injected intravenously over a period of a few minutes. Complete urine collections as judged from constancy of the creatinine were obtained for 3 days after each injection. A sample of the combined urine was counted for ^{14}C and ^{3}H content. Water obtained by distillation from a frozen specimen of another urine aliquot was counted for tritium content. To the remainder of the combined 3-day urine collection of β-glucuronidase, 300 units/ml of urine was added and the mixture was incubated at pH 5.0 in acetate buffer at 38° for 5 days. The urine at pH 5.0 was extracted continuously with ether for 48 hr. The ether extract was washed with ice-cold 9% sodium bicarbonate solution saturated with sodium chloride, with saturated sodium chloride solution, and finally with a very small amount of water. The residue has the designation "steroid extract." After removing an aliquot for counting, the "steroid extract" was separated into its components by means of two countercurrent distributions in different solvent systems as described previously (Gallagher *et al.*, 1958). By this means essentially homogeneous fractions containing 2-methoxyestrone, estrone, and estriol were obtained. In addition, a fraction containing a mixture of estradiol and 2-hydroxyestrone and another containing 16-epiestriol and the various ring D ketols were also obtained.

Inert carrier was added to the appropriate radioactive fractions and the mixture recrystallized until the final three crystallizations exhibited constant specific activity and isotope ratio within the counting error of ±5%. Four crystallizations usually sufficed. Estrone and 2-methoxyestrone were recrystallized from ethanol and methanol; estriol was purified as the triacetate by recrystallization from hexane and a small amount of acetone; estradiol was acetylated, and the diacetate was purified by preparative thin layer chromatography on silica gel with 7:3 cyclohexane–ethyl acetate and recrystallized from petroleum ether (bp 30–60°). 16-Epiestriol was purified as the acetonide. The mixture of carrier and appropriate countercurrent distribution fractions was dissolved in 10 ml of dry, freshly distilled acetone, 5 ml of acetone saturated with dry HCl was added, and the solution was stored overnight. The reaction mixture was poured on ice and water and extracted three times with chloroform. The organic extract was washed with 5% sodium bicarbonate solution and with water, dried, and evaporated. The residue was purified by preparative thin layer chromatography in ethyl acetate and the zone corresponding to the epiestriol acetonide was eluted. The product, mp 182–185°, was recrystallized from acetone–hexane.

Location of Tritium in Metabolites. ESTRONE. A portion of the estrone of constant specific activity was refluxed in 1 N aqueous sodium hydroxide solution for 1 hr under nitrogen. After cooling and acidification with concentrated hydrochloric acid, the estrone precipitate was filtered, washed with water, and crystallized from ethanol to give material of undiminished specific activity with respect to ^{14}C, but lacking any significant tritium counts.

ESTRADIOL. Estradiol of constant specific activity with respect to both isotopes was dissolved by shaking in 0.1 N aqueous NaOH and a small excess of benzoyl chloride was added. After shaking for 10 min at room

temperature the precipitate that formed was filtered, washed well with water, and dried. Without further purification the estradiol-3-benzoate was dissolved in 10 ml of acetone and cooled to 0°, and 2 drops of 8 N chromic acid (Bowers *et al.*, 1953) was added. After standing for 15 min at 5°, the reaction mixture was diluted with water and extracted into chloroform, which was washed with 5% sodium bicarbonate and then water. After drying and removing the solvent, the residue of estrone benzoate was recrystallized from acetone–hexane to give a product with undiminished specific activity and unchanged isotope ratio. When this material was refluxed in 1 N NaOH in 50% aqueous ethanol for 3 hr the estrone that was isolated upon acidification showed undiminished ^{14}C specific activity, but retained no tritium radioactivity.

ESTRIOL. Estriol triacetate of constant specific activity was dissolved in dry tetrahydrofuran and treated with excess $LiAlH_4$. After stirring for 1 hr at room temperature the excess reagent was destroyed by cautious addition of water. More water was then added followed by acidification with dilute sulfuric acid. The estriol was isolated by extraction with chloroform and removal of the solvent. The crude estriol weighing 30 mg (0.104 mmole) was dissolved in 0.5 ml of pyridine and treated with 22 mg of acetic anhydride (0.215 mmole). After standing overnight the reaction was worked up on the usual manner and the crude acetylated mixture was submitted to preparative thin layer chromatography on silica gel in the system 7:3 ethyl acetate–cyclohexane. The zone corresponding to estriol 3,16-diacetate was eluted to give 28 mg of material which, without purification, was dissolved in 5 ml of acetone and treated with 3 drops of the chromic acid reagent at 0°. After standing for 15 min at 5°, water was added and the oxidation product was extracted into chloroform. After removing the solvent, the residue was recrystallized from acetone–hexane to give 21 mg of 16α-hydroxyestrone diacetate, mp 158–162°, identical in all respects with the authentic material (Biggerstaff and Gallagher, 1957). The specific activity and isotope ratio of the diacetate remained essentially unchanged after two more successive crystallizations.

The remainder of 16α-hydroxyestrone diacetate was allowed to stand for 72 hr in 10 ml of 0.1 N NaOH in 50% aqueous methanol. After acidification with 5% sulfuric acid, the product was extracted with $CHCl_3$, dried, and evaporated. The residue was acetylated overnight and the product was recrystallized from acetone–petroleum ether to give 5.8 mg of 16-ketoestradiol diacetate which showed unchanged specific activity with respect to ^{14}C but exhibited no significant tritium activity. Subsequent recrystallization did not alter these results significantly.

Counting Procedures. All the counting was carried out on a Packard-Tricarb liquid scintillation counter. Each product was counted in triplicate and sufficient counts were allowed to accumulate to permit an accuracy of ±5%.

Results

In Table I are recorded the $^{14}C/^{3}H$ ratios of various urinary metabolites isolated following the administration of estradiol-16β-^{3}H and estradiol-16α-^{3}H to the same subject at different times. With estradiol-16β-^{3}H no significant loss of tritium occurred in the formation of estrone, estradiol, and 2-methoxyestrone. This

TABLE I: Subject SO.

	Estradiol-16β-^{3}H		Estradiol-16α-^{3}H	
	$^{14}C/^{3}H$	$\Delta^{3}H$ (%)	$^{14}C/^{3}H$	$\Delta^{3}H$ (%)
Dose	2.30	...	0.62	...
Estrone	2.37	−3	0.71	−13
2-MeO-estrone	2.22	+3	0.67	−7
Estradiol	2.20	+4	0.63	−2
Estriol	2.42	−5	3.10	−80
16-Epiestriol	21.0	−89	0.55	+13

demonstrated that the β-hydrogen at C-16 remained intact during the biotransformation to these compounds as well as in the subsequent isolation procedures. This fact lends authority to the data for estriol and 16-epiestriol. The isotope ratios for these compounds clearly show that all the tritium was retained in the 16α-hydroxy compound, while essentially all the isotope was lost from the epimeric 16β-hydroxy triol. To eliminate the possibility that the tritium in the isolated metabolites was now located at other than the 16β position, perhaps by way of an enzymatically catalyzed hydride shift, it was necessary to define the location of the isotope in these compounds. Exposure of the isolated estrone to aqueous alkali resulted in total loss of tritium; hence it must have been present on C-16, the only enolizable position. Oxidation of estradiol to estrone, after protecting the phenolic hydroxyl as the benzoate, failed to remove any isotope; hence it could not have been present at 17α. Alkali treatment of the estrone benzoate derived from estradiol again resulted in complete loss of isotope. The location of tritium in the isolated estriol was determined by selective acetylation to the 3,16-diacetate followed by oxidation to 16α-hydroxyestrone diacetate. As shown in Table II all the tritium present in the original estriol was retained in the 17-keto compound. Further, all ^{3}H was lost when 16α-hydroxyestrone diacetate was converted to 16-ketoestradiol with alkali. These transformations define the position of the isotope solely at 16β in the estriol and the other metabolites derived from estradiol-16β-^{3}H.

The carbon/tritium ratios of the metabolites isolated following the administration of 16α-tritium-labeled estradiol to the same subject are also given in Table I. There was a greater loss of tritium from the 17-keto steroids, estrone and 2-methoxyestrone, in this study

TABLE II: Subject SO.

		Estradiol-16β-^{3}H		Estradiol-16α-^{3}H	
		cpm/mg[a]	^{14}C/^{3}H	cpm/mg	^{14}C/^{3}H
Estriol triacetate	^{14}C	1560	2.35	3456	3.12
	^{3}H	665		1112	
16α-Hydroxyestrone diacetate	^{14}C	1548	2.46	3420	3.25
	^{3}H	630		1050	
16-Ketoestradiol diacetate	^{14}C	1510	63	3424	260
	^{3}H	24		13	

[a] All specific activities are corrected to free estriol.

TABLE III: Subjects MR and BY.

	MR Estradiol-16β-^{3}H		BY Estradiol-16α-^{3}H	
	^{14}C/^{3}H	Δ^{3}H (%)	^{14}C/^{3}H	Δ^{3}H (%)
Dose	0.98	...	1.78	...
Estrone	1.02	−4	2.38	−25
2-MeO-estrone	0.99	−1	2.30	−22
Estradiol	0.92	+6	1.76	+1
Estriol	1.07	−8	8.63	−78
Epiestriol	8.20	−88	1.52	+17

than was found in the comparable metabolites derived from estradiol-16β-^{3}H. This is consistent with the greater lability of the 16α proton of a 17-keto steroid under enolizing conditions (Fishman, 1966), and would indicate that loss of isotope occurred during isolation rather than *in vivo*. This view is supported by the fact that estradiol retained all the tritium within the error of measurement. Since estradiol is rapidly converted to estrone and in turn regenerated from that metabolite in the body (Fishman *et al.*, 1960), and since estradiol cannot enolize during the isolation procedure, it follows from the unaltered isotope ratio that the hydrogen isotope in the ketonic metabolites was retained under *in vivo* conditions.

A considerable portion, but not all, of the tritium from estradiol-16α-^{3}H was lost from the urinary estriol. Based on the chemical synthesis of the deuterium-labeled estradiol (Fishman, 1965), introduction of the 16α isotope is somewhat less stereospecific than that in the 16β orientation. From nuclear magnetic resonance evidence with the deuterium compounds 80–85% steric purity of the administered 16α-^{3}H-estradiol may be expected and this would then account for the retention of some tritium in the isolated estriol. Indeed the retained tritium was shown to be present at C-16β and at no other site by the same procedures used in the 16β-^{3}H study, and the results are also shown in Table II. Thus, in contrast with estradiol-16β-^{3}H the *in vivo* transformation of the 16α-labeled hormone to estriol was accomplished with loss of all the isotope present at the 16α orientation.

The foregoing results support the nuclear magnetic resonance evidence (Fishman, 1965) that the administered 16α-^{3}H-estradiol contained about 20% of 16β-^{3}H-estradiol. On this basis, then, the epimeric 16-epiestriol should lose *ca.* 20% of its tritium, but in fact a gain in tritium was evident. This does not appear to be due to random error since it was reproduced almost exactly in another study (Table III). It does not appear useful to invoke an isotope effect as the reason for this gain since no similar isotope effect was observed in the comparable hydroxylation leading to estriol from the 16β-^{3}H substrate. Conceivably, the additional tritium in 16-epiestriol may be present at C-17 where it was introduced during the reduction of the 17 ketone in 16β-hydroxyestrone in the presence of tritium available from the biosynthesis of estriol. Since the amount of estriol formed far outweighed that of epiestriol, sufficient tritium might be locally available at the cellular sites of transformation to show the observed effect. Starting with estradiol-16β-^{3}H the situation is reversed, in that not enough tritium would be available from the formation of epiestriol to show any significant incorporation in the isolated estriol. Unfortunately, it was impossible to study these possibilities by location of the position of the additional tritium in 16-epiestriol, since there was insufficient labeled material for the necessary chemical manipulations.

In order to ensure that these results did not represent a unique individual phenomenon, or a male characteristic, but were of a general nature, examination of the metabolites produced from estradiol-16α-^{3}H and -16β-^{3}H was repeated in two different women subjects. The results obtained, listed in Table III, are completely in accord with those seen in the man SO. The larger loss of tritium from ketonic metabolites of subject BY after estradiol-16α-^{3}H emphasizes the random nature of this loss, in accord with its artifactual origin.

The retention of tritium in estriol and its loss in 16-epiestriol derived from 16β-tritioestradiol, and the reverse situation in these compounds derived from 16α-tritioestradiol, define the stereochemistry of the enzymatic hydroxylation at C-16. It conforms to that found at other chemically inactive sites in the molecule in that replacement of hydrogen is involved. The adjacent 17-keto group, therefore, does not affect the stereochemical course of the hydroxylation. The results do not exclude the possibility that the 16α and 16β hydroxylations proceed *via* two different enols obtained by stereospecific abstraction of 16α and 16β hydrogens, respectively. They do, however, exclude a common enol form that serves as the substrate for both 16α and 16β hydroxylations.

This demonstration of the stereochemical course of 16-hydroxylation eliminates from consideration any 16-oxo compounds as intermediates in the *in vivo* biosynthesis of either estriol or 16-epiestriol. The 16α- and

16β-hydroxyestrones appear to be the evident precursors of the estriols. From these ketols, reduction of the ketone at C-17 would readily lead to estriol and epiestriol. This sequence, however, does not account for the demonstrated presence of 16-ketoestradiol (Layne and Marrian, 1958) and 16-ketoestrone (Serchi, 1953; Slaunwhite and Sandberg, 1956) among the products of estrogen biotransformation. The 16-ketoestradiol and 16-ketoestrone isolated from human urine must, therefore, result from oxidation of 16-hydroxylated end products or intermediates and not as normal precursors in the biosynthetic pathway to the estriols. Retention of most of the 16β-^{3}H in this study clearly implies that loss of *both* hydrogens from C-16, such as oxidation to a 16-ketone would require, is not a major biochemical process in man. Furthermore, it can be concluded from the results of this study that the transformation of 16-ketoestradiol to estriol and 16-epiestriol, and the formation of epiestriol from estriol which have been observed both *in vivo* (Levitz *et al.*, 1958, 1960; Nocke *et al.*, 1961; Stimmel, 1958) and *in vitro* (Breuer *et al.*, 1958, 1959; Breuer and Knuppen, 1958), represent quantitatively insignificant pathways or that these results were obtained under conditions incompatible with the *in vivo* catabolism of estradiol in man.

Table IV shows the excretion of radioactivity in urine and the distribution of the carbon and tritium counts obtained in the steroid fraction for the respective estradiol-16β-^{3}H and -16α-^{3}H studies in subject SO. Tritium was also measured in the urine water. The difference in tritium content of the total body water following the administration of 16β-^{3}H- and 16α-^{3}H-labeled hormones is striking.

With subject SO the amount of tritium excreted and the fact that nearly all of it was in organic combination after metabolism of the 16β-^{3}H-labeled substrate are fully in accord with expectation. The small amount of tritium present as ^3H_2O can be reconciled with the quantity of 16-epiestriol produced from the precursor and is reflected accurately in the 3% difference between the recovery of ^{3}H and ^{14}C in the steroid extract. Furthermore the recovery of ^{14}C and ^{3}H isotopes was the same (*i.e.*, 35% of each administered isotope failed to be accounted for). Therefore, with the exception of 16-epiestriol formation, the molecules of estradiol with the 16β hydrogen isotope were treated *in vivo* indistinguishably from those with ^{14}C at C-4. The situation however was quite different following the administration of the 16α-^{3}H-labeled hormone. In this case, since estriol is the only known major 16α-substituted metabolite, it would be expected that the amount of tritium appearing in body water should reflect quite closely the amount of estradiol transformed to estriol. This was not the case. Approximately 8% of the administered hormone was recovered as urinary estriol with the ^{14}C label, whereas 55% of the administered tritium was recovered in the body water. Thus, *ca.* 45% of the estradiol had undergone biochemical attack at the 16α orientation and was not recovered in the urine. Similar results were obtained with the other two subjects MR and BY, and these are recorded in Table V. Again following estradiol-16β-^{3}H administration the total recovery of tritium (50%) approximated that of the ^{14}C-labeled material (42%), while in the estradiol-16α-^{3}H study nearly all the administered tritium (90%) was recovered, in contrast to a 72% recovery of ^{14}C radioactivity. Compared to the previous studies (Table IV), it is significant that the higher tritium content in the body water in this 16β-^{3}H study with subject MR was accompanied by an unusually low ^{14}C excretion in the urine; the lower body water tritium in the 16α-^{3}H study with subject BY was reflected in a higher ^{14}C recovery in the urine.

TABLE IV: Subject SO.

		Estradiol-16β-^{3}H		Estradiol-16α-^{3}H	
		cpm × 10^{-6}	Dose (%)	cpm × 10^{-6}	Dose (%)
Dose	^{14}C	9.30	...	1.86	...
	^{3}H	4.03	...	3.00	...
Total body ^3H_2O	^{3}H	0.18	4.5	1.65	55.0
Urinary solids	^{14}C	6.05	65.0	1.13	60.5
	^{3}H	2.58	64.0	1.44	48.2
Steroid extract	^{14}C	4.56	49.0	0.94	50.5
	^{3}H	1.85	46.0	1.23	41.0
Estriol	^{14}C	0.74	8.0	0.15	8.1
	^{3}H	0.31	7.7	0.05	1.7

TABLE V: Subjects MR and BY.

		MR Estradiol-16β-^{3}H		BY Estradiol-16α-^{3}H	
		cpm × 10^{-6}	Dose (%)	cpm × 10^{-6}	Dose (%)
Dose	^{14}C	1.71	...	1.96	...
	^{3}H	1.80	...	1.11	...
Total body ^3H_2O	^{3}H	0.20	11.1	0.25	23.4
Urinary solids	^{14}C	0.72	42.0	1.42	72.5
	^{3}H	0.71	39.5	0.74	66.5
Steroid extract	^{14}C	0.54	31.6	1.12	57.0
	^{3}H	0.52	29.0	0.59	53.0
Estriol	^{14}C	0.17	10.0	0.07	3.6
	^{3}H	0.17	9.4	0.009	0.8

It has been observed by Sandberg and Slaunwhite (1957), and confirmed in unpublished results from these laboratories, that the total metabolite recovery in patients with complete external biliary fistulas after estradiol administration approximated 100%; *ca.* 50% of the estradiol metabolites were excreted in bile and *ca.* 50% were in urine within the first 24 hr. The most immediate interpretation of our results in the present study is that the ^3H in body water, derived from 16α-^3H, represented estriol that has been excreted in bile, not returned to the general circulation and effectively lost from analysis. Alternatively the metabolite or metabolites that have lost tritium may represent transformation product(s) not as yet defined.

Fortunately, the evidence now available is much in favor of the second alternative. Sandberg and Slaunwhite (1965), in a very careful study, have shown that preformed estriol is excreted in the bile in lesser amount than the metabolites derived from estrone or estradiol. Moreover, this conjugated biliary estriol was efficiently reabsorbed; only a very small portion was excreted in stool and most of this "enterohepatic" estriol appeared promptly in urine. Further to the point, estriol glucosiduronate neither appeared in bile nor was the conjugate cleaved *in vivo*. These facts strongly suggest that the "missing" metabolite(s) are 16α-substituted product(s) other than estriol.

It is certain that the "missing" metabolites are not excreted in urine during the 3-day collection time used in these studies. In earlier studies both from these laboratories (Beer and Gallagher, 1955) and from Sandberg and Slaunwhite (1957), fecal excretion of radioactivity derived from labeled estradiol and estrone was small, of the order of 5% of the amount given. The considerably greater quantity of the "missing" products demonstrated in the present study would suggest that the intestinal route was not the means of disposal or that the time of study was not sufficiently prolonged to accumulate any appreciable quantity of the "missing" metabolites. The detection and characterization of these metabolites present an important and intriguing problem for further studies.

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