Aromatic Ring Hydroxylation of Estradiol in Man*

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ABSTRACT: Estradiol specifically labled with tritium at C-2 was synthesized and administered to man together with estradiol-4-^{14}C.

Tritium displaced by metabolism at C-2 appeared in the body water and its rate and extent was compared with that following estradiol-17α-^{3}H administration. The isotope content of urinary metabolites obtained after enzyme and acid hydrolysis showed that 2-oxygenated compounds represented 22% of the dose. These metabolites were biosynthesized with complete displacement of tritium. The tritium in the body water was 33% of the dose and exceeded that derived from excreted metabolites. It is suggested that the excess body water tritium is derived from additional 2 hydroxylation the products of which are not excreted in the urine. These results in conjunction with those previously obtained with estradiol-16α-^{3}H indicate that all of the missing fraction of administered estradiol is metabolized at 16α but only a part of it is also metabolized at C-2. The hydroxylation at C-2 proceeds without any isotope effect suggesting that the removal of the hydrogen is not the rate-determining step. However, a small isotope effect is operating in the conjugation process resulting in a tritium enrichment of the sulfate conjugates. An additional isotope effect also serves to increase the tritium content of the excreted estradiol in contrast to all the other metabolites.

Following the administration of labeled estradiol to man a varying but substantial portion of the radioactivity fails to appear in the urine (Zumoff *et al.*, 1968). Since fecal excretion of estrogens is small (Beer and Gallagher, 1955; Sandberg and Slaunwhite, 1957) the fate of the missing material is a challenge for investigation. A previous study from this laboratory (Fishman *et al.*, 1966) employed estradiols stereospecifically labeled with tritium at 16α and 16β. It was demonstrated that the missing metabolites were substituted at 16α but not 16β since the excreted 16α-oxygenated metabolites plus the fraction of the dose that was not recovered almost precisely accounted for the tritium liberated from estradiol-16α-^{3}H. The present work was initiated to find out the extent of metabolism at C-2 involved in the pathway leading to the missing compounds. This is a logical site for such transformation since cleavage of the aromatic ring A could be a likely form of degradation and loss of estradiol metabolites.

Estradiol labeled specifically with tritium at C-2 was prepared and administered to man. The fate of the hydrogen isotope was monitored in body fluids as well as in the urinary metabolites and distinguished from the remainder of the molecule which was labeled with carbon-14. This procedure was also expected to shed light on other aspects of the enzymic *in vivo* formation of estrogen catechols.

Materials and Methods[1]

Carrier estrone, estradiol, and estriol were obtained commercially and were homogenous by thin-layer chromatography and melting point determination. Carrier 2-hydroxyestrone,[2] 2-methoxyestrone, 2-hydroxyestradiol, 16-ketoestradiol, and 16α-hydroxyestrone were prepared in this laboratory and their homogeneity was confirmed by melting point, thin-layer chromatography, and spectral determinations. 17β-Estradiol-4-^{14}C (31.8 mCi/mmole) was obtained commercially. 17β-Estradiol-17α-^{3}H (63 μCi/mg) was prepared in this laboratory as previously described (Fishman *et al.*, 1961). Both labeled substrates were better than 97% pure by reverse isotope dilution.

Preparation of 17β-Estradiol-2-^{3}H. 2-Bromoestradiol was prepared and carefully purified to homogeneity as described (Utne *et al.*, 1968). The pure material melted at 195–197° and its diacetate exhibited two aromatic proton singlet absorptions in the nuclear magnetic resonance spectrum at 410 and 450 Hz. In order to ensure that the catalytic reduction of 2-bromoestradiol proceeds without rearrangement, the preparation of 17β-estradiol-2-^{2}H was carried out first. A solution of 50 mg of 2-bromoestradiol in 10 ml of 10% methanolic KOH was reduced with deuterium gas over 10% palladiumized charcoal for 16 hr (Coombs and Roderick 1968). After the catalyst was removed by filtration, the solution was diluted with 15 ml of cold water and the pH was adjusted to 6 with 5% hydrochloric acid. Extraction with chloroform which was washed with water, dried, and evaporated gave 24 mg of residue which by thin-layer chromatog-

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[1] The nuclear magnetic resonance spectra were obtained on a Varian A60 instrument. Chemical shifts are in Hertz downfield from tetramethylsilane as an internal standard. The solvent used was deuteriochloroform. Counting was carried out on a Packard Tri-Carb liquid scintillation counter. Each sample was counted in triplicate and sufficient counts were allowed to accumulate to permit an accuracy of ±2%. The counting efficiency for ^{14}C was 79.8% and for ^{3}H 46.2%.

[2] Common names used are: 2-hydroxyestrone, 2,3-dihydroxyestra-1,3,5(10)-trien-17-one; 2-methoxyestrone, 2,3-dihydroxyestra-1,3,5(10)-trien-17-one 2-methyl ether; 2-hydroxyestradiol, 2,3,17β-trihydroxyestra-1,3,5(10)-triene; 16-ketoestradiol, 3,17α-dihydroxyestra-1,3,5(10)-trien-16-one; 16α-hydroxyestrone, 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one; 2-bromoestradiol, 2-bromoestra-1,3,5(10)-triene-3,17β-diol.

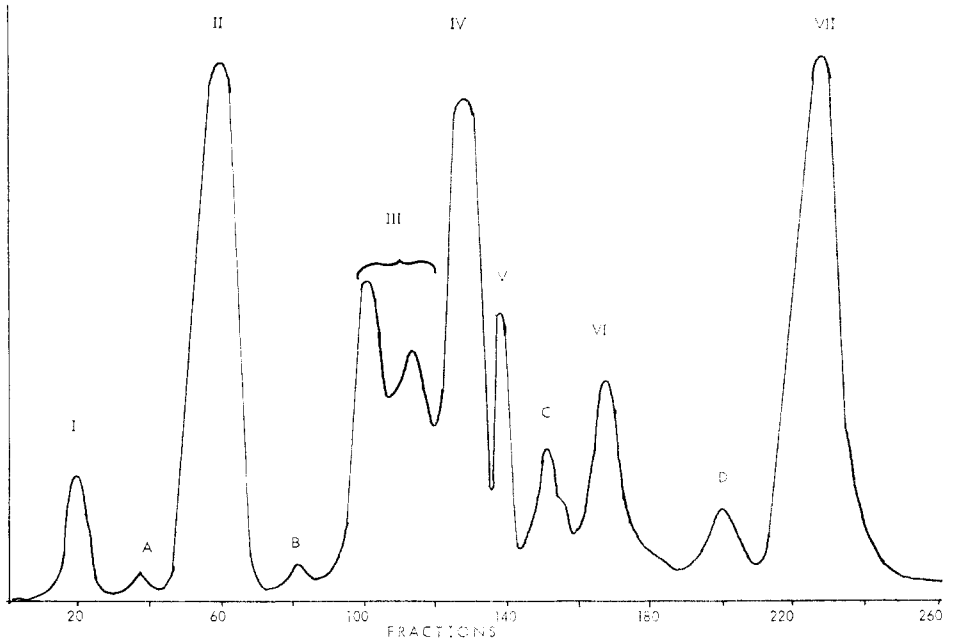

FIGURE 1: Isotope ratios of known and unknown radioactive areas from partition chromatography of enzyme hydrolysate.

	Known	$^3H/^{14}C$	Unknown	$^3H/^{14}C$
I	2-Methoxyestrone	0.3	A	0.8
II	Estrone	4.9	B	1.6
III	Estradiol and 2-hydroxyestrone	1.8	C	2.7
			D	0.6
IV	16α-Hydroxyestrone and 16-ketoestradiol	4.9		
V	16β-Hydroxyestrone	4.8		
VI	16-Epiestriol	4.7		
VII	Estriol	4.9		

raphy corresponded to estradiol-17β. Acetylation and recrystallization from acetone–petroleum ether gave pure 17β-estradiol-2-^{2}H diacetate. The nuclear magnetic resonance spectrum of this material showed only two aromatic proton singlets at 407 and 435 Hz, indicating the location of the deuterium in the aromatic ring to be C-2 exclusively.

A 10-mg sample of 2-bromoestradiol in 5 ml of 10% methanolic KOH was reduced with 25 Ci of tritium over 10% palladiumized charcoal for 2 hr. After filtration, the solvent was removed and the residue treated three times with 5% aqueous acetic acid. The residue was then taken up in acetone and submitted to preparative thin-layer chromatography on silica gel. Development was first in 95% chloroform and 5% acetonitrile, and then in 50% ethyl acetate and 50% cyclohexane. The zone coincident with standard estradiol was eluted with ethanol. The eluted material contained 12.25×10^9 cpm.

Proof of Location of ^3H and Homogeneity of Product. A portion of the above estradiol-2-^{3}H containing 3.16×10^5 cpm was added to 25.2 mg of inert estradiol, which was then recrystallized three times from benzene. The specific activities of the three crops were 12,150, 12,070, and 12,210 cpm per mg, respectively, indicating a purity of 96.8% for the estradiol-2-^{3}H.

To confirm the location of the tritium in the labeled compound, the above estradiol-2-^{3}H containing 1250 cpm/mg was brominated to 2-bromoestradiol (Utne *et al.*, 1968). After purification the product was crystallized from ethanol to show successive specific activities of 140, 75, 60, 58, and 59 cpm per mg. The 2-bromoestradiol therefore retained only 4.7% of the tritium originally present and 95.3% of the isotope in estradiol-2-^{3}H was located at C-2.

Subjects Body Water Volumes. Subject MA was a 26-year-old woman with porphyria. Her creatinine excretion was 1.14 g/24 hr, lean body mass was 46.5 kg, and calculated body water volume was 34.0 l. (Miller and Blyth, 1952).

Subject LO was a 60-year-old man with a depressive reaction. His creatinine was 1.06 g/24 hr, lean body mass 43.6 kg, and calculated body water 31.8 l. His body water volume was determined by injecting 1×10^6 cpm of [^{3}H]H_2O and obtaining the specific activity of the water derived from a plasma sample withdrawn 90 min later. The specific activity of the lyophilized water was 30 cpm/ml to give a body water volume of 33.4 l.

17β-Estradiol-17α-^3H Experiment. Before administration to subject MA, estradiol-17α-^{3}H (63 μCi/mg) 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 2 min. The weight of injected solution was determined by difference and gave the dose administered as 25.8×10^6 cpm. Plasma samples were obtained at intervals during the first 6 hr and were immediately frozen. Two urine collections of 0–6 hr and 6–30 hr were collected. Water obtained by lyophilization

TABLE I: Isotope Excretion Following Estradiol-2-^{3}H-4-^{14}C Administration.

Dose:	32.55		6.58		4.95
	^{3}H[a]		^{14}C		
	cpm × 10^{6}	% Dose	cpm × 10^{6}	% Dose	^{3}H/^{14}C
		Urine			
Day 1	10.50	32.3	2.66	40.5	3.95
Day 2	3.13	9.6	1.13	16.9	2.76
Day 3	1.28	3.9	0.56	8.5	2.28
Day 4	0.45	1.4	0.18	2.8	2 50
Total	15.36	47.2	4.53	68.7	3.40
		Hydrolysates			
Glucuronidase	11.32	34.8	3.35	51.0	3.38
Solvolysis	1.88	5.8	0.45	6.8	4.20
Total	13.20	41.6	3.80	57.8	

[a] The urinary ^{3}H counts are corrected for those present as H_2O.

of the plasma and urine collections was counted for ^{3}H content.

17β-Estradiol-2-^{3}H Study. Estradiol-2-^{3}H (50 mCi/mg), 32.55 × 10^{6} cpm, and estradiol-4-^{14}C (31.8 mCi/mmole), 6.58 × 10^{6} cpm, were mixed and administered to subject LO intravenously in propylene glycol. The tritium to carbon-14 ratio of the dose was 4.95. Plasma samples were obtained at intervals for the first 6 hr, with one final sample at 24 hr. Urine was collected at 24-hr intervals for 4 days. Aliquots of each urine collection were counted for ^{3}H and ^{14}C content. Water obtained by lyophilization of plasma and urine aliquots was counted for ^{3}H content.

To an aliquot of the pooled urine collection 36.4 mg of inert 2-hydroxyestrone was added, and the urine was incubated with β-glucuronidase (300 units/ml) pH 5 in acetate buffer for 5 days. The urine at pH 5 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 water. The residue, the enzymic hydrolysate, was counted for ^{14}C and ^{3}H content. The spent urine was adjusted to 4.5% (v/v) sulfuric acid content, an additional 23.7 mg of carrier 2-hydroxyestrone was added, and the mixture was extracted continuously with ether for 72 hr. The ether extract was washed as previously and the residue, the acid hydrolysate, was counted for ^{14}C and ^{3}H content.

Each extract was separately subjected to gradient elution partition chromatography on acid-washed Hyflo Super Cel column with 90% aqueous methanol as the stationary phase and trimethylpentane with a gradient of dichloroethane as the mobile phase according to Engel *et al.* (1961). Fractions of 10 ml were collected in an automatic fraction collector. The radioactive areas were located by counting aliquots with a windowless gas-flow counter. Each area of radioactivity was pooled and an aliquot was counted for ^{3}H and ^{14}C content (Figure 1).

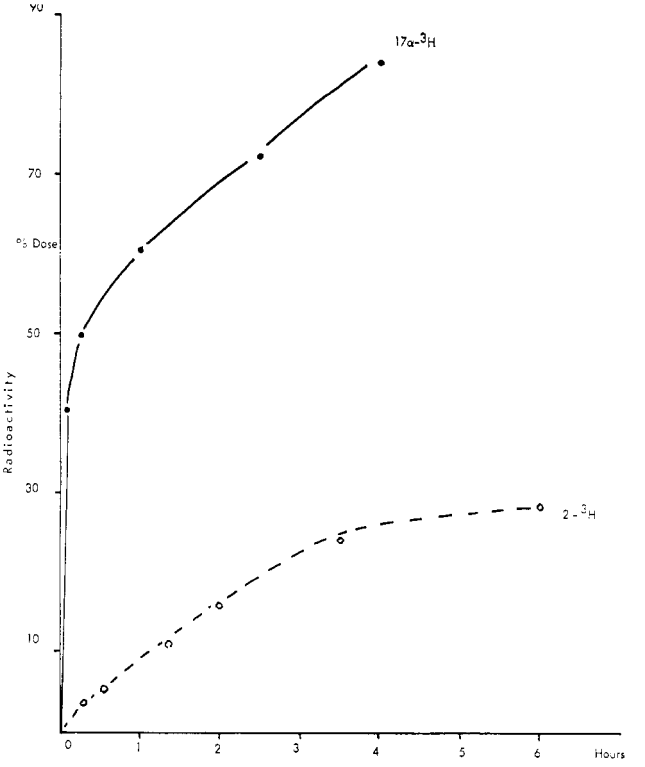

FIGURE 2: Rate of appearance of tritium in body water following estradiol-17α-^{3}H and estradiol-2^{3}H administration.

To each pooled radioactive area the appropriate inert carrier steroid was added, the mixture was acetylated and following purification by preparative thin-layer chromatography the derivative was recrystallized to constant specific activity and isotope ratio. To the area containing 2-hydroxyestrone and estradiol, carrier estradiol was added, the mixture was acetylated, and estradiol diacetate and 2-hydroxyestrone diacetate were separated by preparative thin-layer chromatography before recrystallization (Fishman, 1963). The region of the enzyme hydrolysate containing 16α-hydroxyestrone and 16-ketoestradiol was divided into two equal portions. One of the above two carriers was added to each, acetylated, and recrystallized to constant isotope content (Fishman *et al.*, 1967).

Results

The rate of release of tritium from C-2 as measured by the appearance of tritium in plasma water is shown in Figure 2. The rate appears to be relatively slow, with a half-time of approximately 2 hr. The reaction is complete after 24 hr, since the urine water of later collections did not show any further ^{3}H enrichment. For comparison the kinetics of estradiol-17α-^{3}H oxidation was similarly followed in another patient and the results are also given in Figure 2. This reaction is much faster with a half-time of less than 5 min and was essentially complete (86%) in 4 hr.

The excretion of both ^{3}H and ^{14}C radioactivity in the sequential urine collection is listed in Table I. The ^{3}H values represent only organically bound isotope. As expected in the first 3 days a progressively smaller percentage of tritium

TABLE II: ^3H as H_2O in Plasma and Urine.

	Estradiol-2-^3H		Estradiol-17α-^3H	
	cpm/ml	% Dose[a]	cpm/ml	% Dose
Plasma (min)				
5			308	41.5
15	32	3.3	390	50.8
30	50	5.1		
60			478	62.7
75	92	9.5		
120	136	13.9		
150			554	74.0
240	260	26.5	656	86.0
360	280	28.7	612	
1440	320	32.8		
Urine (hr)				
0–6			573	
6–30			601	
0–24	294			
24–48	324			
48–72	302			
72–96	291			

[a] As calculated from total body water volume.

TABLE III: Isotope Content of Enzyme-Hydrolyzed Metabolites of Estradiol-2-^3H-4-^{14}C.

	^{14}C			
Metabolite	cpm × 10^6	% Dose	$^3H/^{14}C$	Δ% ^3H
2-Methoxyestrone	0.17	2.6	0.30	−94
2-Hydroxyestrone	0.98	14.9	0.29	−94
Estrone	0.41	6.2	4.89	−1
Estradiol	0.17	2.7	5.17	+4
Estriol	0.35	5.3	4.88	−1
16α-Hydroxyestrone	0.16	2.4	4.84	−2
16-Ketoestradiol	0.05	0.8	4.83	−2
Unidentified areas	0.18	2.7	0.3–1.0	
Polar eluate	0.13	2.0	1.60	

relative to ^{14}C was excreted daily. The total tritium so excreted corresponded to 47.2% of that originally administered, while the same value for the carbon isotope was 68.7%. The isotope ratios found in the urine reflect this disparity in that the value was uniformly lower than that of the material administered. Table I also contains the isotope content of the metabolites from both the enzyme and the acid solvolysates representing the glucosiduronates and "sulfate" conjugates, respectively. It is apparent from the isotope ratios that the solvolyzed metabolites contained more tritium than the glucosiduronates, possible due to a lesser proportion of 2-oxygenated metabolites in this form.

The tritium contained in body water is given in Table II. The body water volume was measured by isotope dilution analysis as 33.4 l. This value times the specific activity of the plasma water at 24 hr gives a value of 10.6×10^6 cpm of ^3H in the total body water, equivalent to 32.8% of that administered. The tritium contained in the body water following estradiol-17α-^3H administration is also given in Table II. In this case the body water volume was calculated from the lean body mass formula (Miller and Blyth, 1952) to give a body water tritium content in accord with previous results showing a rapid and essentially complete oxidation of estradiol to estrone (Fishman *et al.*, 1961).

The isotope distribution in the metabolites liberated by glucuronidase hydrolysis is given in Table III. All of the values for the identified compounds are based on reverse isotope dilution, derivative formation, and recrystallization to constant specific activity and isotope ratio. The carbon-14 values serve to quantitate the metabolic product, while the tritium to carbon-14 ratio permits calculation of tritium loss or gain. The 2-oxygenated metabolites exhibit a 94% loss of tritium, equivalent to all the isotope located in this position. All the other metabolites with the exception of estradiol showed virtual total retention of tritium or a slight loss of questionable significance. Estradiol exhibited a small gain of tritium. As this isotope ratio was constant in every recrystallization the small increase is considered significant and is discussed later.

Similar values for solvolyzed metabolites are given in Table IV. The 2-oxygenated metabolites again showed a 94% loss of tritium, but in every other instance these metabolites exhibited a higher tritium content than the corresponding compounds liberated by enzyme hydrolysis. Estradiol again had a higher tritium content than the other solvolyzed metabolites. The slight tritium increase can be satisfactorily explained by the presence of an isotope effect which is discussed later. It is noteworthy that the unidentified polar eluates from the chromatography of both the acid- and enzyme-hydrolyzed extracts exhibited an isotope ratio suggesting a high content of 2-oxygenated constituents.

Table V contains a balance sheet of the administered and isolated isotopes in their various forms. The total 4-day urine collection contained 68.7 and 47.4% of the administered ^{14}C and ^3H, respectively. The ^{14}C-labeled metabolites excreted contained 2-oxygenated compounds equivalent to at least 22% of the dose. The tritium lost in the formation of these compounds when added to that still attached at C-2 in the other excreted metabolites equals 69.4% of that in the dose, a value in excellent agreement with the total ^{14}C excreted. The tritium liberated from C-2 by 2 hydroxylation appears in the body water, but the actual amount of tritium so present exceeds that expected being equal to 32.8% of the dose. Thus, 10.8% of the tritium administered found in the body water has no counterpart in the ^{14}C metabolites excreted and is of unknown origin. The portion of the administered ^{14}C and ^3H which remains missing is therefore 31.3 and 19.8%, respectively. The central fact emerging from this compilation is that although of the two isotopes administered more of the ^3H than of the ^{14}C can be found, a substantial portion of the ^3H still remains missing and is unaccounted for.

The partition chromatography of the hydrolysis extracts produces a series of discrete radioactive areas most of which represent known compounds. A number of relatively minor

TABLE IV: Isotope Content of Acid-Hydrolyzed Metabolites of Estradiol-2-^{3}H-4-^{14}C.

Metabolite	^{14}C cpm × 10^{6}	^{14}C % Dose	^{3}H/^{14}C	Δ% ^{3}H
2-Methoxyestrone	0.024	0.4	0.30	−94
2-Hydroxyestrone	0.175	2.7	0.30	−94
Estrone	0.065	1.0	5.17	+4
Estradiol	0.012	0.2	5.36	+8
Estriol	0.054	0.8	5.14	+4
Polar eluate	0.055	0.8	1.86	

TABLE V: Balance Sheet of Isotopes Following Estradiol-2-^{3}H-4-^{14}C Administration.

	% Dose ^{14}C	% Dose ^{3}H
Urine	68.7	47.2
2-Oxy metabolites	22.0	22.0[a]
Body water		32.8
Excess in body water		10.8
Total recovered	68.7	80.0
Missing	31.3	20.0

[a] In body water derived from excreted 2-oxymetabolites.

radioactive areas, however, have not been identified. The isotope ratio of each radioactive zone shown in Figure 1, serves to give clues to the nature of these unknowns since a low ^{3}H to ^{14}C ratio indicates the presence of carbon-2 substitution.

Discussion

The results of this study bear on a number of aspects of estradiol metabolism in man. These are discussed below under separate headings.

Recovery of ^{3}H and ^{14}C Isotopes. The urinary excretion of ^{14}C radioactivity is only 67% of that injected. Approximately one-third of these excreted metabolites or 22% of the dose are 2-oxygenated compounds. The tritium released by their formation appears in the body water but the quantity found exceeds that which could be derived from 2-oxygenated metabolites which are excreted. The excess tritium present in the body water, equal to about 11% of that administered, is most likely derived as the result of additional C-2 hydroxylation, the products of which are not excreted in the urine and hence are not measured. The kinetics of the appearance of tritium in the body (Figure 2) indicates that this is indeed the case. The simple hyperbolic shape of the curve suggests a homogeneous enzyme reaction and not separate processes being involved in the release of tritium from C-2.

Although the recovered ^{3}H radioactivity was proportionally greater than ^{14}C, 20% of the administered tritium still remained missing. This is in contrast to the situation with estradiol-16α-^{3}H where all of the isotope can be accounted for (Fishman *et al.*, 1966). From this it may be concluded that all of the missing part of injected estradiol is metabolized at 16α but only one-third of the missing metabolites are also metabolized at C-2. An important consequence of these results is that cleavage of ring A of estradiol *via* the 2,3-catechols is not an important pathway of estrogen degradation in man since the products would have yielded the tritium located at C-2 to the body water in quantities in excess of those found. The portion of estradiol metabolized both at C-2 and C-16 can be postulated at its simplest as 2-hydroxyestriol and its methyl ether. The latter has been found in human urine in minor quantities (Fishman and Gallagher, 1958), but it is of course possible that most of what is formed is not excreted by this route.

A consequence of the findings in this experiment is to lend support to the reverse isotope dilution procedures employed in quantitating radioactive 2-hydroxyestrone in urine (Fishman, 1963). The fact that the calculated value of 2-hydroxy-metabolites compensates almost exactly for the difference between the total content of ^{14}C and ^{3}H in the excreted metabolites suggests strongly that this figure is fully representative of the 2-hydroxy compounds actually excreted.

Kinetics of ^{3}H Release from C-2. The rate of hydroxylation of estradiol at C-2, as measured by the appearance of tritium in body water, appears to follow simple kinetics. The reaction has a half-time of about 2 hr and is essentially complete at 6 hr. In contrast, the oxidation of estradiol-17α-^{3}H is a much faster reaction with a half-time of less than 5 min. The dehydrogenation must actually be an even faster process since the presence of a substantial isotope effect (Adams *et al.*, 1965) serves to retard the oxidation of the 17α-^{3}H-containing molecules. Since it has been demonstrated that estrone and not estradiol is the substrate for 2 hydroxylation (Fishman *et al.*, 1960), the rapid formation of estrone should provide early an adequate substrate for 2 hydroxylation. Other considerations such as entry into site of reaction and release of tritium into body water undoubtedly also play a role in the observed kinetics.

NIH Shift. Analysis of the tritium location in the administered tritiated estradiol showed that 95% of its tritium content was located at carbon-2. The tritium analysis of the excreted 2-hydroxy and 2-methoxy metabolites showed these to have lost 94% of the isotope and therefore the 2 hydroxylation proceeds with total loss of the hydrogen at C-2 and there is no shift of the displaced hydrogen to carbon-1. This agrees with the proposed mechanism for the NIH shift and the hypothetical semiquinone intermediate proposed for *ortho* hydroxylation (Guroff *et al.*, 1967) in other instances of catechol formation. The lack of any NIH shift in the 2 hydroxylation of estradiol permits the use of 2-tritio substrates and the consequent tritium release as a convenient and reliable method of monitoring this enzymic reaction.

Isotope Effects. ON AROMATIC HYDROXYLATION. Estrone is the substrate for 2 hydroxylation (Fishman *et al.*, 1967) and in the case studied at least 25% of the available estrone is so metabolized. The tritium content of the excreted estrone conjugates does not differ significantly from that of the administered dose and therefore the hydroxylation at C-2

proceeds with no isotope effect. This is in contrast to the case of the estradiol dehydrogenase where replacement of the 17α-hydrogen with an isotope produces a profound isotope effect (Adams *et al.*, 1965). The lack of an isotope effect in aromatic hydroxylation is strong evidence that in this enzymic reaction loss of the hydrogen atom is not the rate-determining step. It would appear that the addition of oxygen, in whatever form it occurs, is the crucial consideration. This would also account for the lack of any tritium exchange on the part of the unreacted substrate.

ON CONJUGATION. Although no primary isotope effect is observed in the metabolism of estradiol-2-^{3}H there is evidence for the presence of a small secondary effect connected largely with conjugation. The isotope ratios of the enzyme-hydrolyzed and -solvolyzed metabolites listed in Tables III and IV were obtained by reverse isotope dilution and in every case the isotope ratios of the three final crystallizations agreed within ±1%. Inspection of these figures reveals two facts of interest. The solvolyzed metabolites in every instance exhibited a higher ^{3}H to ^{14}C ratio than those liberated by glucuronidase. In both series of compounds estradiol is distinctly higher in tritium than the other metabolites. The small tritium increase noted in the metabolites excreted as sulfates is particularly significant since the acid conditions used in solvolysis would be expected to result in a loss of tritium (Coombs and Roderick, 1968). The tritium increase can be the result of either a positive isotope effect in sulfate conjugation or a negative one in glucosiduronation. Since the latter conjugation predominates over sulfation, the observable effects of either isotope effect, *i.e.*, the isotope ratio, would be concentrated in the smaller portion of the substrate that is excreted as sulfates and the difference in isotope ratio from that of the dose would be more apparent in the sulfated than in the glucosiduronated metabolites. Such an isotope effect induced by the presence of tritium at C-2 is not unreasonable when one considers that the isotope is located *ortho* to the conjugating phenolic hydroxyl and is therefore in a position to exert influence not only by its proximity but also *via* electronic effects transmitted by resonance and hence may discriminate in the enzymic esterification of the phenol.

The tritium enrichment of estradiol over all other metabolites is somewhat more difficult to rationalize since its metabolic transformation is limited to oxidation at C-17, a site remote from the isotope at C-2. A possible explanation is that estradiol is subject to selective protein binding. If the tritium at C-2 exerts a negative isotope effect on this binding then the unbound and available for excretion estradiol will be somewhat tritium enriched. The above speculations suggest that these isotope effects albeit small may be of significant theoretical and practical importance and deserve further investigation.

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