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Oxidative Metabolism of Estradiol*†

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The metabolism of the estrogenic hormone is oxidative in nature in contrast to the reductive pathway for the neutral steroid hormones. It has been postulated that estrone is the precursor of estriol on the evidence that the required ketol intermediates are normal metabolites of the estrogenic hormone (2–4). However, it is equally true that enzymatic hydroxylation of chemically unreactive methylene groups is a common feature of steroid biochemistry (5), and thus estradiol might serve as the immediate precursor of estriol with the ketols formed by subsequent oxidation. To clarify this problem we have examined the metabolism of estrone and estradiol simultaneously in the same human subject.

For this purpose, estradiol labeled with tritium in ring B and estrone-16-C^{14} were dissolved together and administered intravenously to three different patients. The ratio of weight as well as isotope of the two hormones was varied in each of the studies. Urine and blood collections were obtained at successive intervals immediately after the injections and the isotopic content of the metabolites was examined after purification to radiochemical homogeneity. The results of the three studies were completely concordant. It was shown that the rate of conversion of estrone to estradiol was slow, whereas the reverse reaction was quite rapid. It seems clear that estrone rather than estradiol is the major precursor of estriol, 2-methoxyestrone, and 16-epiestriol.

EXPERIMENTAL PROCEDURE

Material and Methods—Estrone-16-C^{14}, specific activity 2.7 μc per mg,[1] was shown to be at least 97% pure by paper chromatography and reverse isotopic dilution analysis. Estradiol-6,7-H^3, with a specific activity of about 7 mc per mg, was obtained as the diacetate from Dr. Marcel Gut of the Worcester Foundation. The diacetate was reduced with $LiAlH_4$ and the reaction product was chromatographed on paper for 60 hours in the system propylene glycol-toluene. The area of radioactivity coincided with that of the standard of authentic estradiol chromatographed simultaneously. This region was eluted with methanol and a portion was removed for reverse isotopic dilution with carrier estradiol. Samples from successive recrystallizations were counted with a windowless gas-flow counter and with a liquid scintillation counter. Both methods showed the labeled material to be 98% estradiol.

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† A preliminary report on some of this work has been published (1).

[1] Obtained from Charles E. Frosst and Company, Montreal.

Estrone and estradiol for carrier studies were obtained from commercial sources and were recrystallized to constant melting point. Estriol, 2-methoxyestrone, and 16-epiestriol were synthesized in these laboratories and were purified to constant melting point. The infrared spectra of all of the compounds were identical with pure reference steroids. Commercial propylene glycol was redistilled under reduced pressure and only the center cut was used.

All subjects were women who were catheterized, and urine collections were made at specified intervals starting with ½ hour after injection. Portions of each injection solution were removed and the ratio of C^{14} to H^3 determined.

The urine collected at each interval was processed individually by the procedure used in these laboratories (6). Glucuronidase[2] incubation for 5 days at pH 5 was followed by continuous extraction with ether; the ether extract was washed with 9% $NaHCO_3$ solution. The crude phenolic and neutral extract thus obtained was subjected to a 99-tube countercurrent distribution in System I composed of 70% methanol-carbon tetrachloride. The distribution was examined by measurement of radioactivity. Tubes 70 to 99 were combined and redistributed through 99 tubes in System II composed of ethanol-water-ethyl acetate-cyclohexane, 1:1:1:1. This distribution in the two systems resolved the estrogen metabolites of interest into well defined areas with the following peak tubes: System I, 2-methoxyestrone in tube 22 and estrone in tube 56; System II, estriol in tube 28 and estradiol in tube 62. In addition, a region with a peak at tube 48 contained the epiestriol and the ring D ketol metabolites.

The appropriate regions in the countercurrent distribution were mixed with carrier steroid followed by crystallization to constant specific activity. Estriol and epiestriol were converted to the triacetates before counting because of the insolubility of the free steroids in the scintillant mixture. Aliquots of the peak countercurrent tube of each metabolite and each of the adjacent tubes were counted before carrier addition.

With Subject R, Table III, the countercurrent region containing 16-epiestriol (tubes 37 to 56) was chromatographed on paper in the system toluene-isooctane-methanol-water, 15:5:16:4. Standards of epiestriol and 16-ketoestradiol were chromatographed simultaneously. The 16-epiestriol area, as determined by the radioactivity measured with an Aquebogue scanner, was eluted and inert 16-epiestriol added. After acetylation the material was recrystallized from methanol to constant specific activity and the ratio of C^{14} to H^3 determined.

[2] Known as Ketodase and obtained from the Warner-Chilcott Laboratories, a division of Warner-Lambert Pharmaceutical Company.

The collected blood (60 cc) was diluted with 10 volumes of 1:1 ethanol-acetone mixture, filtered, and the precipitate washed well with ethanol-acetone. The filtrate and washings were concentrated to dryness and the residue was made to 500 cc with water and extracted continuously with ether for 24 hours. The ether extract was washed with a 9% sodium bicarbonate solution saturated with sodium chloride. The dry residue (40,700 c.p.m.) was dissolved in 70% ethanol and washed with petroleum ether. The aqueous ethanol was removed and the "free" steroids (33,700 c.p.m.) were subjected to countercurrent separation in Systems I and II. The aqueous residue from the ether extraction of the blood was adjusted to pH 5 with acetic acid, and 0.1 volume of acetate buffer (pH 5.0) containing 11 mg of Versene (ethylenediaminetetraacetate) per ml and 1 ml of toluene was added. The solution was incubated with limpet enzyme (7) for 5 days at 37° and continuously extracted with ether for 48 hours. The ether extract was washed with sodium bicarbonate solution and the ether was removed. The dry residue (66,000 c.p.m.) was submitted to countercurrent separation.

The isotope ratios of each metabolite were measured differentially with a Packard Tricarb liquid scintillation counter equipped with the Blau modification (8). It should be noted that the measured isotope ratios are not the true ratio of the two isotopes but rather the ratio of that fraction of the total disintegrations from each of the two isotopes measured by the instrument under the experimental conditions employed.

RESULTS

Estrone-Estradiol—The first study was primarily designed to establish whether the approach was feasible. The mixture administered contained estrone-16-C^{14} and estradiol-17β,6,7-H^3 in a mass ratio of 330 and a measured isotope ratio of 0.54. Estrone obtained from the urine during the first half hour after administration had a measured isotope ratio of 0.59 and this value remained virtually constant for estrone throughout the study (Table I). During the first half hour the excreted estradiol contained so little C^{14} that this isotope could not be measured. Even by the 12th hour the measured isotope ratio for estradiol was different from the estrone isolated during this period; by the second day after the injection, however, the excreted estradiol had a ratio of C^{14} to H^3 indistinguishable from the injected mixture.

In the second study a mixture containing equal weights of the two hormones and a measured isotope ratio of 1.16 was administered to a different patient (Table II). Again estrone during the first half hour after administration had nearly the isotope ratio of the injected mixture since the ratio of C^{14} to H^3 for the metabolite was 1.32. By the end of the first hour this value was experimentally indistinguishable from that of the injected mixture. As in the initial study, the estradiol during this same interval had a measured ratio of 0.05 and 0.28 for the first two 30-minute periods. Even during the ninth hour after administration of the mixture of hormones, estradiol had an appreciably lower measured isotope ratio than either estrone or the injection mixture.

In the third experiment a mixture containing a 7 to 1 mass ratio of estrone to estradiol and a measured C^{14} to H^3 ratio of 0.92 was administered. Again the estrone rapidly approached the isotope ratio of the injected mixture since during the first half hour after administration the ratio of C^{14} to H^3 was 1.15 for the urinary metabolite. By the end of the second half hour the isotope ratio of estrone was indistinguishable from the injection mixture. In contrast, estradiol with C^{14} to H^3 0.08 and 0.39 for the first two half hours had a clearly different isotope composition. Not until the sixth hour after administration had the estradiol become isotopically indistinguishable from the estrone samples (Table III). During the time of greatest disparity in the measured isotope ratio of these metabolites, *i.e.* from zero through the fourth hour, the amount of either isotope lost from the body by excretion was too little (2% of the dose) to have had any measurable effect upon the isotope ratio of the compounds within the body.

Estriol—The estriol samples were almost indistinguishable isotopically from the corresponding estrone samples in the earliest collection containing sufficient estriol radioactivity to permit differential isotope analysis. There was no significant change in the measured ratio at later times. The ability to detect estriol in patients W and R, at the times recorded was possible only because of the large amount of radioactivity given in these studies.

Other Metabolites—The intentionally large amount of radioactivity in the third study permitted isolation of 16-epiestriol early in the study when differences in isotope content between estrone and estradiol were still substantial. The isotope ratio

TABLE I

Subject M (age 43, multiple sclerosis)

Dose: Estrone-16-C^{14}, 1 mg, 2.7 μc; estradiol-6,7-H^3, 3 μg, 20 μc in 1 ml of propylene glycol; measured C^{14} to H^3 = 0.54.

Mid-time	C^{14} to H^3			
	Estrone	Estradiol	Estriol	2-Methoxy-estrone
hours				
$\frac{1}{4}$	0.59*	0*		
$\frac{3}{4}$	0.56*	0.12*		
$1\frac{1}{2}$	0.51*	0.15*		
$2\frac{1}{2}$	0.49*	0.18*	0.50*	
4	0.50	0.26	0.42*	
9	0.53	0.43	0.44*	0.57*
36	0.56	0.57	0.53	0.55*

* Only peak tubes counted.

TABLE II

Subject W (age 57, carcinoma of breast, oophorectomized and adrenalectomized)

Dose: Estrone-16-C^{14}, 2 mg, 5.4 μc; estradiol-6,7-H^3, 2 mg, 20 μc in 2 ml of propylene glycol; measured C^{14} to H^3 = 1.16.

Mid-time	C^{14} to H^3			
	Estrone	Estradiol	Estriol	2-Methoxy-estrone
hours				
$\frac{1}{4}$	1.32	0.05		
$\frac{3}{4}$	1.10	0.28		
$1\frac{1}{2}$	1.15	0.34	0.98	1.10*
$2\frac{1}{2}$	1.08	0.49	1.06	
$3\frac{1}{2}$	1.14	0.65	1.05	0.86*
$4\frac{1}{2}$	1.09	0.80	1.03	
$7\frac{1}{2}$	1.15	0.93	1.11	0.97*

* Only peak tubes counted.

TABLE III

Subject R (age 60, carcinoma of breast)

Dose: Estrone-16-C^{14}, 10.9 mg, 29.5 μc; estradiol-6,7-H^3, 1.5 mg, 150 μc in 3 ml of propylene glycol; measured C^{14} to H^3 = 0.92.

Mid-Time	C^{14} to H^3				
	Estrone	Estradiol	Estriol	2-Meth-oxyestrone	Epiestriol
hours					
¼	1.15	0.08			
¾	0.84	0.39	0.72	0.82*	
1½	0.81	0.62	0.77	0.82*	0.77
2½	0.83	0.66	0.81	0.87	0.76
4½	0.85	0.74	0.80	0.91*	
7½	0.87	0.81	0.86	0.85*	
Blood collection at ½ hour					
Free	4.00	0.04			
Conjugated	0.85	0.48			

* Only peak tubes counted.

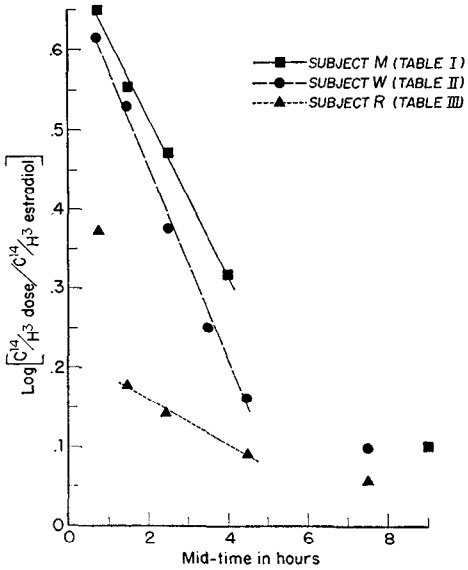


FIG. 1. Kinetics of the isotope composition of estradiol

at this earliest practicable interval shows that this compound, too, had a measured isotope ratio very similar to estrone, estriol, and the mixture injected. The same was true for 2-methoxyestrone.

The "free" blood steroids at 30 minutes in Subject R (Table III) did not have the same isotopic composition as the conjugated steroids. This is presumed to result principally from the persistence of small amounts of the injected estrogens not as yet withdrawn into tissues. The important fact is that the conjugated steroids, which had unquestionably entered the tissues as evidenced by formation of the glucosiduronate, showed essentially the same isotope composition as the urinary estrogens measured about 30 minutes later.

From the radioactivity present in the blood sample it was calculated that 3.8% of the dose was in the estimated 4-liter volume. A large portion (66%) of this radioactivity was conjugated, *i.e.* these were metabolites released from the tissues. Thus, only 30 minutes after the time of administration, some 98% of the relatively large amount of "free" hormone, 12.4 mg, had been removed from the blood.

In order to compare the studies more directly, the isotope ratio values of estradiol in each experiment have been divided into the corresponding final isotope ratio. The logarithms of the figures thus obtained are plotted *versus* mid-time in Fig. 1, and straight lines are obtained for the earlier portion of all studies. The reactions cease to be first order during the late collection periods because other interconversions begin to show their effects. Although the first two experiments show lines with essentially identical slopes, the third experiment exhibits a quite different slope. In addition, the earliest point in this experiment is not on a straight line. A very reasonable explanation is the high radioactivity of the administered estradiol which, if cleared in small amount without conjugation, would contribute sufficient tritium to the metabolized and conjugated urinary estradiol to result in an abnormally low C^{14} to H^3 ratio.

As evident from Fig. 1 (9), some form of first order reaction kinetics applies to the change in isotope ratio of estradiol and of necessity to the reversible oxidation-reduction reaction. Recent studies of these estrogens with isolated enzyme systems (10–12) strongly suggest that a pyridine nucleotide-linked enzyme system is involved in the reaction. Therefore, circumstances must exist under which such a second order reaction would appear to exhibit first order kinetics. This is possible if, in the sites where principal metabolism of these hormones occur, the concentration of TPNH, DPNH, TPN, and DPN are very large relative to the concentration of estrogen. Under such circumstances there will be no significant change in the concentration of the coenzymes during the course of reaction. Since the pH surely remains constant, the pertinent variable becomes the reciprocal alteration in the concentration of estrone and estradiol and a pseudo first order reaction is obtained.

DISCUSSION

These three studies, in which widely different mass ratios of estrone-C^{14} and estradiol-H^3 were injected, showed that urinary estrone and conjugated blood estrone at the earliest time examined had virtually the same measured isotope ratio as the injection mixture. Urinary estradiol and conjugated blood estradiol, in contrast, contained relatively little C^{14}-radioactivity at this time. At a much later time the measured isotope ratio of estradiol approached that of the mixture injected and was virtually identical with this value during the following day.

The only possible means by which tritium could appear in estrone was the oxidation *in vivo* of the administered estradiol. The blood and urine measurements at the earliest sampling showed that at least 90% of the tritium injected had been incorporated into estrone.

The early achievement of a C^{14} to H^3 ratio in urinary estrone, virtually but not completely identical with that of the injected mixture of steroids, indicates that estradiol is initially and almost completely converted to estrone. Thus, Reaction I is rapid

(I) Estradiol → Estrone

even when the mass ratio of estrone was 333 to 1 of estradiol. Conversely, the later appearance of a C^{14} to H^3 ratio approaching that of the mixture injected indicates that this estradiol was formed secondarily from estrone. Therefore, Reaction II

(II) Estradiol ← Estrone

is slow. These results are the same for the three widely varying mass ratios studied. A necessary consequence of these rate differences is that estrone occupies a central position in estrogen metabolism.

The purpose of this study was to obtain information about the immediate precursor of estriol (16α-hydroxylation), epiestriol (16β-hydroxylation), and 2-methoxyestrone (hydroxylation to form a catechol and subsequent methylation). It seems clear, from comparison of the isotope ratios of these metabolites at the earliest times that they appeared in measurable quantities, that their isotopic composition closely approximated that of estrone and deviated considerably from estradiol. The simplest explanation of these facts is that estrone was the immediate precursor that was oxidized in the A and D rings. The interesting conclusion, then, is that estrone is the only direct metabolite of estradiol and all the other estrogens isolated represent further alterations of estrone.

The conclusions drawn from this study are summarized in Scheme 1. It is evident that 16-ketoestradiol (13, 14) and 16-ketoestrone (15) present problems with respect to their route of biogenesis. It is possible that 16-ketoestrone may be derived from oxidation of either 16α- or 16β-hydroxyestrone and selective reduction at C-17 of the diketone might explain the presence of the third known ring D ketol metabolite. Experiments to study the pathway involved in the production of these compounds are now in progress.

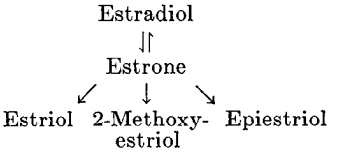
The rapidity of two reactions observed in this study deserves comment. The fact that in less that 30 minutes more than 98% of a large unphysiological amount of hormone had been removed from the circulation implies a highly effective mechanism for the capture of both estrone and estradiol by tissues. The speed of this process together with the very rapid oxidation of estradiol may be interpreted as an indication that:

1. A single organ with a characteristic concentrating mechanism and oxidative enzyme system dominates, in a quantitative sense, the metabolic fate of the estrogenic hormone. This does not imply that this organ (probably liver) is the sole site for the immediate localization of estrogen since it is clear that many tissues must be able to obtain a supply of the hormone. However, there must either be a great difference in the capacity of one organ and its oxidative enzyme system or a similar enzyme must be widely distributed in organs and tissues.

2. The systems responsible for the further metabolic alteration of the estrogen molecule, *e.g.* transformation to estriol, must have a similar tissue distribution since so little estradiol or estrone circulates in the blood.

3. The biological activity of estrone may be due to the relatively slow reaction by which it is transformed to estradiol. On the other hand, considering the speed with which estrone is produced and the fact that all other metabolites are formed from estrone, it may be that this substance is in fact the circulating hormone as distinct from the product secreted by the glands. In this view, the reaction that regenerates estradiol may be a means for the conservation and prolongation of estrogenic action.

4. Since in some species, particularly the rat, estradiol has a greater biological activity than estrone, it may be suggested that the rapid oxidation observed in the human may not occur to the same extent in these species. It is perhaps significant to note in this connection that reduction of estrone to estradiol-17α is characteristic of many animals (16) but is an insignificant reaction in the human, if indeed it occurs at all.



SCHEME 1.
Routes of estrogen metabolism in man

These considerations which arise from this study of the transformation of two related steroids, simultaneously administered, provide a basis for many other investigations of the dynamics of estrogen metabolism. It is believed that further study of these basic biochemical facts may well provide the means for understanding how these hormones achieve their profound effects upon tissues and the metabolic systems under hormonal control

SUMMARY

A mixture of estrone-16-C^{14} and estradiol-6,7-H^3 in varying mass and isotope ratio was administered to three patients. Comparison of the measured isotope ratio in estrone and estradiol in successive urine collections demonstrated that oxidation of estradiol to estrone must be more rapid than reduction of estrone to estradiol. From the measured isotope ratio of other metabolites isolated it was concluded that estriol, epiestriol, and 2-methoxyestrone are derived from estrone and not from estradiol.

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REFERENCES

1. FISHMAN, J., BRADLOW, H. L., AND GALLAGHER, T. F., *J. Am. Chem. Soc.*, **81**, 2273 (1959).
2. MARRIAN, G. F., LOKE, K. H., WATSON, E. J. D., AND PANATTONI, M., *Biochem. J.*, **66**, 60 (1957).
3. LAYNE, D. S., AND MARRIAN, G. F., *Biochem. J.*, **70**, 244 (1958).
4. BROWN, B. T., FISHMAN, J., AND GALLAGHER, T. F., *Nature (London)*, **182**, 50 (1958).
5. FRIED, J., THOMA, R. W., PERLMAN, D., HERZ, J. E., AND BORMAN, A., in G. PINCUS (Editor), *Recent progress in hormone research, Vol. 11*, Academic Press, Inc., New York, 1955, p. 149.
6. BEER, C. T., AND GALLAGHER, T. F., *J. Biol. Chem.*, **214**, 335 (1955).
7. DODGSON, K. S., LEWIS, J. I. M., AND SPENCER, B., *Biochem. J.*, **55**, 253 (1953).
8. BLAU, M., *Nucleonics*, **15**, 90 (1957).
9. GLASSTONE, S. T., *Textbook of physical chemistry*, 2nd edition, Van Nostrand, New York, 1946, p. 1046.
10. TALALAY, P., HURLOCK, B., AND WILLIAMS-ASHMAN, H. G., *Proc. Natl. Acad. Sci., U. S.*, **44**, 862 (1958).
11. HAGERMAN, D. D., VILLEE, C. A., AND ENGEL, L. L., *J. Biol. Chem.*, **234**, 2031 (1959).
12. LANGER, L. J., ALEXANDER, J. A., AND ENGEL, L. L., *J. Biol. Chem.*, **234**, 2609 (1959).
13. LEVITZ, M., SPITZER, J. R., AND TWOMBLY, G. H., *J. Biol. Chem.*, **222**, 981 (1956).
14. WATSON, E. J. D., AND MARRIAN, G. F., *Biochem. J.*, **61**, XXIV (1955).
15. SLAUNWHITE, W. R., JR., AND SANDBERG, A. A., *Arch. Biochem. Biophys.*, **63**, 478 (1955).
16. KLYNE, W., AND WRIGHT, A. A., *Biochem. J.*, **66**, 92 (1957).