Directive O Methylation of Estrogen Catechol Sulfates*

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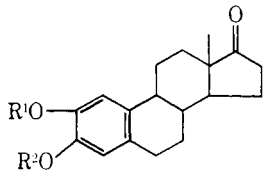
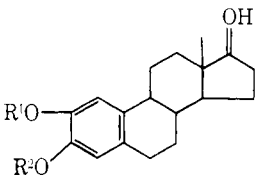
ABSTRACT: O methylation of 2-hydroxyestrone with rat liver homogenate is random with both possible monomethyl ethers being formed in nearly equal quantities.

In contrast, the methylation of the 2- and 3-sulfate esters of 2-hydroxyestrone is directed preferentially to the unconjugated phenol. The unequal effect of the two monosulfates is considered to be due to the more rapid enzyme hydrolysis of the 2-sulfate prior to methylation. "Purified" rat liver catechol-*O*-methyl transferase preparation which lacks sulfatase activity methylates the free 2-hydroxyestrone but not the sulfated substrates. This indicates the necessity for concurrent sulfate removal for the effective O methylation of the adjacent phenol. The successive 2-hydroxylation and O methylation of estradiol by rat liver homogenate leads nearly exclusively to 2-methoxyestrone and suggests that the process proceeds *via* the sequence: estradiol → estrone → estrone sulfate → 2-hydroxyestrone 3-sulfate → 2-methoxyestrone.

The catechol, 2-hydroxyestrone[1] (Ia), is the major



Ia, $R^1 = R^2 = H$
b, $R^1 = CH_3$; $R^2 = H$
c, $R^1 = H$; $R^2 = CH_3$
d, $R^1 = R^2 = SO_3^-$



IIa, $R^1 = R^2 = H$
b, $R^1 = CH_3$; $R^2 = H$
c, $R^1 = H$; $R^2 = CH_3$
d, $R^1 = H$; $R^2 = SO_3^-$
e, $R^1 = SO_3^-$; $R^2 = H$

metabolite of the female sex hormone in man (Fishman *et al.*, 1960a,b; Fishman, 1963). A portion is further converted *in vivo* to the 2-methyl ether (Ib) (Kraychy and Gallagher, 1957) with little or no evidence for the formation of the isomeric 3-methyl ether. This selective O methylation is particularly significant because the two phenolic groups have virtually indistinguishable chemistry (Fishman and Liang, 1968). Similarly the *in vivo* O methylation of the important catechol amines involves nearly exclusively the *m*-phenolic hydroxyl (Axelrod *et al.*, 1958). In sharp contrast to these *in vivo* results, *in vitro* methylation with rat liver catechol-*O*-methyl transferase is relatively indiscriminate yielding mixtures of monomethylated isomers often in equivalent amounts (Senoh *et al.*, 1959; Daly *et al.*, 1960; Knuppen and Breuer, 1966). This divergence between the results *in vitro* and those in the living animal suggest that additional factors must participate in the methylation of 2-hydroxyestrone *in vivo*. In view of the accumulating evidence of active participation of steroidal sulfate esters in metabolic transformations, the present study was undertaken to explore the possibility that such sulfate conjugates are involved in the selectivity of catechol O methylation in man.

Materials and Methods

Carrier estradiol and 2-hydroxyestrone were homogeneous by thin-layer chromatography and melting point determination. The 2-methyl and 3-methyl ethers of 2-hydroxy estrone were prepared in these laboratories as previously described (Fishman *et al.*, 1960a,b). The preparation of 2-sulfate, 3-sulfate, and 2,3-disulfate esters of catechol estrogens has been described elsewhere (Miyazaki and Fishman, 1968a,b). [^{14}C-methyl]*S*-adenosylmethionine (47.5 mCi/mmole) was purified prior to use by the method of Cantoni (1953).

Enzyme Preparations. Fresh rat liver was homogenized in ice-cold 0.25 M sucrose solution to a final concentration of 20%. The homogenate was centrifuged for 15 min at 1000*g* to remove the cell debris and the supernatant was used for the incubation studies. When estradiol was the substrate and sequential 2 hydroxylation and methylation were required the homogenate was prepared in isotonic potassium chloride. The "purified" enzyme preparation was obtained from this homogenate as described (Axelrod and Tomchick, 1958).

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[1] Trivial names will be used for: 3,17β-dihydroxyestra-1,3,5(10)-triene, estradiol-17β; 2,3-dihydroxyestra-1,3,5(10)-trien-17-one, 2-hydroxyestrone; 2,3-dihydroxyestra-1,3,5(10)-trien-17-one 2-methyl ether, 2-methoxyestrone; 2,3-dihydroxyestra-1,3,5(10)-trien-17-one 3-methyl ether, 2-hydroxyestrone 3-methyl ether; 2,3-dihydroxyestra-1,3,5(10)-trien-17-one 3-sulfate ester, 2-hydroxyestrone 3-sulfate; 2,3-dihydroxyestra-1,3,5(10)-triene 2-sulfate, 2-hydroxyestrone 2-sulfate.

Incubations. The incubations involving catechol or catechol sulfate substrates were carried out in an incubation medium consisting of 5 ml of homogenate, or "purified" enzyme, 1 ml of 0.1 M phosphate buffer, 3 mg of $MgSO_4$ and 7×10^5 cpm of [^{14}C-methyl]*S*-adenosylmethionine. About 150–200 μg of substrate was added and the incubation mixtures were shaken at pH 7.8 in air for 180 min at 37°. Large variations in the quantity of *S*-adenosylmethionine added failed to have any effect on the yield and nature of the methylation products. The incubations were terminated by cooling in an ice bath and then diluted with 20 ml of water and extracted with four 20-ml portions of ether–chloroform (3:1, v/v). The extract was dried over anhydrous Na_2SO_4 and evaporated. The aqueous residues were then made 1 N with respect to perchloric acid and were kept overnight at 49°. They were then extracted with ether (five 30-ml portions), which was dried and evaporated. The amount of radioactivity liberated from the acid hydrolysis was in every instance less than 5% of that present in the initial extract and none of it was associated with methylated estrogens.

The incubations in which estradiol was the substrate, and in which initial enzymatic 2 hydroxylation was required were modified to permit optimum 2 hydroxylation (Marks and Hecker, 1966). The conditions used were 5 ml of rat liver homogenate, 10 μmoles of NADP, 120 μmoles of glucose 6-phosphate, 10 units of glucose 6-phosphate dehydrogenase, 100 μmoles of $MgCl_2$, [^{14}C-methyl]*S*-adenosylmethionine (9×10^5 cpm), and 150 μg of estradiol made up to a final volume of 10 ml with Tris buffer. The rest of the incubation conditions and work up were identical with those described above.

In those incubations where accurate quantitation of the products was required carrier 2-methyl and 3-methyl ethers of 2-hydroxyestrone Ib and Ic (*ca.* 10 mg each) were added to the incubation at termination. Following extraction the residue was taken up in dry tetrahydrofuran and stirred at room temperature for 2 hr with an excess of lithium aluminum hydride. The excess reagent was then destroyed by the cautious addition of water and the mixture was extracted with ether which was dried over Na_2SO_4 and evaporated.

Product Isolation and Quantitation. In the absence of carriers, the incubation extract was streaked on Whatman No. 1 paper impregnated with formamide and developed with cyclohexane for 10 hr (Knuppen and Breuer, 1966). The location of the radioactive peaks was determined by radioactivity scanning and the position was matched with standards of 2-methyl and 3-methyl ethers of 2-hydroxyestrone and 2-hydroxyestradiol which were run concurrently. The relative quantity of the radioactive products was obtained by integration by means of weighing of the excised areas. Repeated attempts to elute the radioactivity from the paper gave at best only a 30% recovery, and hence this could not be used as a quantitative procedure.

Separation of the products by alumina chromatography was used when carriers had been added. Following $LiAlH_4$ reduction the products were adsorbed on a neutral alumina column which was developed with benzene and benzene–ethanol mixtures. Fractions of 10 ml were collected, and every fourth fraction was assayed for radioactivity. The ultraviolet absorption at 286 mμ in ethanol was determined on other selected fractions which had first been taken to dryness. The actual distribution of radioactivity between the two isomers was obtained by combining the fractions containing each, acetylating with acetic anhydride in pyridine and recrystallizing to constant specific activity from petroleum ether (bp 30–60°)–acetone. The identity of each final crystalline product was confirmed by nuclear magnetic resonance spectroscopy.

Radioactive Counting. Samples containing ^{14}C were counted in a Nuclear-Chicago Unilux liquid scintillation counter. Toluene containing 6 g/l. of 2,5-diphenyloxazole and 300 mg/l. of 1,4-bis[2-(5-phenyloxazole)]-benzene was used as scintillant. Aqueous samples were counted in diotol, a solvent composed of 1 l. of dioxane, 1 l. of toluene, 600 ml of methanol, 208 g of naphthalene, 13 g of PPO, and 260 mg of dimethyl-POPOP.

Discussion

The analyses of the products of the O methylation arising from different substrates are listed in Table II. These results were obtained by carrier addition, reduction, separation of the 2- and 3-methoxy isomers IIb and c and finally the recrystallization of the diacetates to constant specific activity. A representative analysis by this procedure is detailed in Table I. These values are more reliable and significant than those obtained initially by integration of the radioactive areas following paper chromatography of the incubation products, but the results are in agreement by both methods. The $LiAlH_4$ reduction simplifies the analysis in that any 17-keto products yield only one set of *O*-methyl isomers. In view of the critical nature of the separation of the isomeric methyl ethers the identity of the crystalline

TABLE I: Incubation of Rat Liver Homogenate with 250 μg of 2-Hydroxyestradiol 3-Sulfate in the Presence of 7×10^5 cpm of [^{14}C]*S*-Adenosylmethonine.

	2-Methoxy (IIb)	3-Methoxy (IIc)
Amount added (mg)	10.14	10.16
Specific activity (cpm/mg)		
Crystallization 1[a]	12,460	2,350
Crystallization 2	12,350	1,620
Crystallization 3	12,360	1,580
Crystallization 4	12,400	1,590
Total counts per minute	125,500	16,100
Yield[b]	17.9	2.3
Ratio	7.8	1

[a] Crystallizations carried out on diacetate. Specific activities are corrected to free compound. [b] Based on [^{14}C]methionine available.

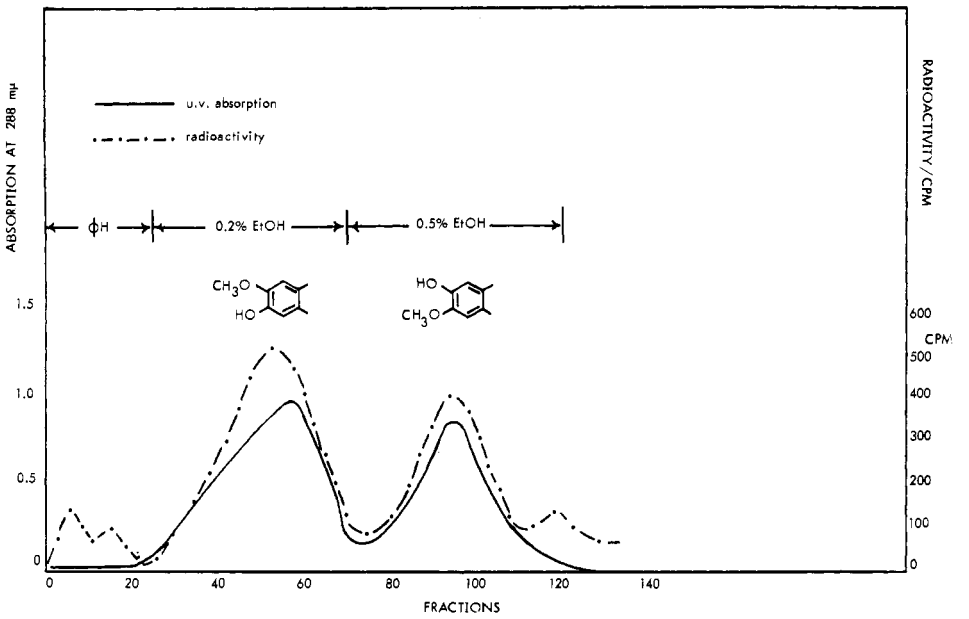

FIGURE 1: Ultraviolet absorption (———) and radioactivity studies (·—·—·).

TABLE II: All Incubations Using 200 μg of Substrate and 7×10^5 cpm [^{14}C]*S*-Adenosylmethionine.

Substrate	2-Methoxy	3-Methoxy	Ratio 2-Methoxy/3-Methoxy
Estradiol	24,300	350	70
2-Hydroxyestrone (Ia)	6,800	5,600	1.2
2-Hydroxyestradiol (IIa)	11,900	10,400	1.1
2-Hydroxyestradiol 3-sulfate (IId)	125,500	16,100	7.8
2-Hydroxyestradiol 2-sulfate (IIc)	37,100	75,500	0.5
2-Hydroxyestrone 2,3-disulfate (Id)	88,000	18,800	4.6
2-Hydroxyestrone[a]	7,500	6,800	1.1

[a] Incubation with "purified" enzyme preparation. All incubations with this enzyme preparation using conjugated substrates failed to yield any O-methylated estrogens.

diacetates from the last crystallization was confirmed by means of nmr spectroscopy in which the chemical shift of the aromatic C-1 and C-4 protons is clearly diagnostic (Fishman and Liang, 1968).

Incubation of 2-hydroxyestrone (Ia) or 2-hydroxyestradiol (IIa) established that these catechols were randomly O methylated *in vitro* giving both possible monomethyl isomers in approximately equal quantities. This confirms results previously obtained by Knuppen and Breuer (1966) using unlabeled materials. In contrast, both monosulfate esters IId and e were methylated selectively with the methylation directed unequally but predominantly to the free, unesterified phenolic hydroxyl. Thus the 2-hydroxy 3-sulfate ester (IId) gave eight times as much 2-methylated as 3-methylated products. The 2-sulfate 3-hydroxy substrate (IIc) was somewhat less selectively methylated but still produced twice as much 3-methoxy as 2-methoxy derivatives. Since sulfatase activity was demonstrably present in the liver homogenate it is reasonable to suppose that methylation at the site of the sulfate conjugate was the result of prior hydrolysis followed by reaction with the free catechol without the selective influence of the sulfate group. The difference in the directive effect of the 2- *vs.* 3-sulfate conjugates suggests that the former was more rapidly cleaved by the enzyme(s) present in the homogenate exposing a larger proportion of the substrate to random unselective methylation. This difference in sulfate hydrolysis rates gains support from the results of incubation with the 2,3-disulfate substrate Id. The 4.5 to 1 ratio of 2-methylated *vs.* 3-methylated products obtained is sufficiently close to the 3 to 1 value expected from a threefold faster hydrolysis of the 2-sulfate which is the difference calculated from the results of the two monosulfate incubations. This difference in the enzyme hydrolysis of the isomeric sulfate esters which are chemically hydrolyzed at identical rates (Miyazaki and Fishman, 1968a,b) raises the possibility of the involvement of two different sulfatases and is being further explored.

The selective *in vitro* O methylation of 2-hydroxyestradiol-3-sulfate (Id) gave the methylated products in a proportion more nearly like that obtained *in vivo*. This convergence of *in vitro* and *in vivo* results is in contrast to the divergence found with the free catechol substrate and suggests strongly the participation of a similar 2-hydroxy 3-sulfate structure in the *in vivo* biotransformations. The 2-hydroxy 3-sulfate conjugate can be envisioned as formed by preferential biosynthesis from the free catechol. A less direct route involves sulfation of estradiol or estrone at C-3 and subsequent hydroxylation at C-2 of the intact sulfate ester. Such a sequence would lead exclusively to the proposed 3-monosulfate catechol isomer. Support for the latter hypothesis is obtained from *in vitro* studies with estradiol as the substrate. The product of the enzymic transformation involving sequential hydroxylation and O methylation was virtually exclusively the 2-methoxy compound. The almost complete absence of 3-methylated 2-hydroxyestrogens suggests that the reaction sequence from the phenol to the 2-methylated catechol estrogen occurs at a subcellular site where substrate and all the necessary enzymes and cofactors are in close proximity. It is clear from the estradiol incubation that en route to the final 2-methoxy product unconjugated 2-hydroxyestrone cannot have any significant lifetime since otherwise the complete sequence must produce significant amounts of the 3-methoxy derivative.

The O-methylated products derived from the sulfated substrates were obtained only as the free compounds. The chemical stability of sulfate esters of guiacols is less than that of catechols (Miyazaki and Fishman, 1968a,b) but it is still sufficient that nonenzymatic hydrolysis during isolation of the products can be excluded. It appears therefore that O methylation is of necessity accompanied by enzymic hydrolysis of the adjacent sulfate conjugate. Experiments performed with the "purified" enzyme preparation (Axelrod and Tomchick, 1958) which is largely devoid of sulfatases bear on this point. Incubation with free 2-hydroxyestrone (Ia) proceeded normally and yielded the 2- and 3-methoxy derivatives in quantities and ratio the same as those obtained with the unfractionated homogenate. None of the sulfated derivatives, however, were effective substrates for O methylation with the "purified" *O*-methyltransferase preparation. From this it appears, that while the sulfate controls the direction of O methylation its removal is necessary for the reaction to take place. This anomaly may be rationalized by the involvement of different methyl transferases for the free catechol and the catechol sulfate substrates. Alternatively, the hydrolysis of the sulfate group and O methylation may be so closely linked in time and space that the directive effect of the conjugate persists after its removal.

Extension of results from this study to the O methylation of catechol amines is of particular interest. Abnormal O methylations of these biogenic amines has been implicated in the etiology of mental disease (Schildkraut and Kety, 1967). If conjugate involvement in the O methylation of these compounds can be demonstrated then the abnormalities in the methylation may have their foundation in the conjugation and deconjugation processes of these compounds.

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