Although the compound which yields fluorescence by reacting with I is not limited to bisulfite as mentioned above, in practical point of view, the present method would permit selective determination of bisulfite in biological materials; because highly interfering compounds, pyrosulfite and hyposulfite, are usually not found in nature, and furthermore, inorganic sulfide and thiols could be eliminated by treating with *p*-chloromercuribenzoic acid prior to addition of I to the sample.

The structure of II is unknown, however, two fluorescent compounds with different mobilities toward the anode (1.6 cm and 2.8 cm) were found by paper electrophoresis (1 M HCOOH, pH 1.75, 36 v/cm, 10 min).

Extension of the present method to fluorometric determination of inorganic and organic thiosulfates (Bunte salts), polythionates *etc.* is now under way. A more complete account of the present procedure will be given in the near future.

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Evidence for O-Methylation of Catechol Estrogen 2-Glucuronoside with Retention of Conjugate in the Rat

It has previously been demonstrated that O-methylation occurred at the nonconjugated phenolic group of catechol estrogen monoglucuronoside in the rat.[1] However, whether the glucuronoside linkage would be retained intact during O-methylation or not still remains unclear. In this paper we wish to present a definite evidence for *in vitro* formation of 2-hydroxyestrone 3-methyl ether 2-glucuronoside with rentention of the inherent sugar moiety.

An initial effort was directed to preparation of 2-hydroxyestrone 2-glucuronoside-^{14}C as a substrate. It is substantiated that the rat, hamster, and guinea pig are capable of catalyzing the glucuronyl transfer exclusively at the C-2 phenolic group of catechol estrogen.[2] Incubation of 2-hydroxyestrone with rat liver homogenate in the presence of uridine diphosphoglucuronic acid-^{14}C afforded the desired 2-hydroxyestrone 2-glucuronoside-^{14}C in a satisfactory yield.

Fresh liver from male Wistar rats (*ca.* 250 g) was homogenized in ice-cooled 0.25 M sucrose solution to a final concentration of 20%. The homogenate was centrifuged for 20 min at 1500×*g* and the supernatant was used for the incubation study. The incubation mixture contained liver homogenate (6 ml), 2-hydroxyestrone 2-glucuronoside-^{14}C (0.73 μCi, 8.3 mg), [^{3}H-methyl]-S-adenosylmethionine (2.5 μCi), magnesium sulfate (4 mg), D-glucaro-1,4-lactone (1 mg), and 0.1 M phosphate buffer (pH 7.5) to make a final volume 8 ml. The mixture was incubated for 90 min at 37°. The incubation mixture was deproteinized with ten-fold volume of ethanol and the supernatant was evaporated *in vacuo*. To the residue was added 2-hydroxy-

1) S. Honma and T. Nambara, Abstract of the 5th Symposium on Drug Metabolism and Action, Shizuoka 1973, p. 85.
2) K.I.H. Williams, *Steroids*, **15**, 105 (1970); I. Yoshizawa, K. Fujimori, and M. Kimura, *Chem. Pharm. Bull.* (Tokyo), **19**, 2431 (1971).

estrone 3-methyl ether 2-glucuronoside (12 mg) as a carrier and the desired fraction was separated by gel filtration on Sephadex LH-20 employing 0.2M acetic acid/60% methanol as an eluant, whereby each fraction was checked by radioactivity counting and colorimetric determination by Kober reaction.

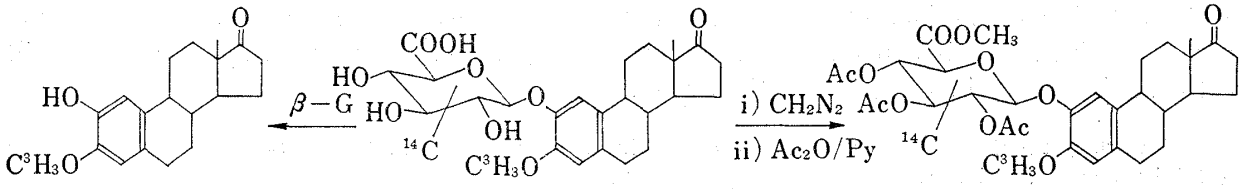

Chart 1

The methylated product was divided into two portions, which were used for derivatization into the acetate-methyl ester and enzymatic hydrolysis, respectively. One of these was treated with diazomethane and then with acetic anhydride–pyridine in the usual manner. To this product was added methyl (3-methoxy-17-oxoestra-1,3,5(10)-trien-2-yl-2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate[3] (11 mg) as a carrier and crystallized repeatedly. The constant isotope ratio was attained as listed in Table I.

Table I. Identification of 2-Hydroxyestrone 3-Methyl-^{3}H Ether 2-Glucuronoside-^{14}C by Reverse Isotope Dilution Analysis[a]

No.	Crystallization from	Specific activity (dpm/mg)		^{3}H/^{14}C
		^{3}H	^{14}C	
1	MeOH	4740	14350	0.330
2	MeOH	3860	15660	0.246
3	MeOH	4150	14960	0.277
4	MeOH	4130	17000	0.243

a) Crystallization was carried out on the acetate-methyl ester.

Table II. Identification of 2-Hydroxyestrone 3-Methyl-^{3}H Ether derived from Its 2-Glucuronoside-^{14}C by Reverse Isotope Dilution Analysis

No.	Crystallization from	Specific activity (dpm/mg)		^{3}H/^{14}C
		^{3}H	^{14}C	
1	benzene–hexane	16460	1170	14.1
2	benzene–hexane	16350	1340	12.2
3	benzene–hexane	16450	1370	12.0

The other portion was submitted to hydrolysis with beef-liver β-glucuronidase (G) (20000 Fishman units) for 48 hr at 37°. The hydrolyzate was extracted with ether, diluted with a carrier, 2-hydroxyestrone 3-methyl ether, and then purified by preparative thin-layer chromatography. The resulting 2-hydroxyestrone 3-methyl ether showed almost the complete loss of ^{14}C radioactivity and was unambiguously characterized by isotope dilution analysis as listed in Table II.

It is evident from these data that catechol estrogen 2-glucuronoside underwent O-methylation at the nonconjugated phenolic group yielding the 3-methyl ether with retention of the

3) S. Honma and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **22**, 687 (1974).

inherent conjugate. It has already been demonstrated that O-methylation of 2-hydroxyestrone 3-sulfate was similarly directed to the unconjugated phenol to afford the 2-monomethyl ether in the rat.[4] This transformation, however, necessitated the concurrent sulfate removal for the effective O-methylation of the adjacent phenol. In view of the accumulating evidences for active participation of the steroidal conjugates the present result on selective O-methylation of the catechol monoglucuronoside is of particular interest. Whether or not the enzyme system involved in this transmethylation is identical with catechol O-methyltransferase will be the subject to be investigated in the future.

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4) M. Miyazaki, I. Yoshizawa, and J. Fishman, *Biochemistry*, **8**, 1669 (1969).