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 (1M 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 retention of the inherent sugar moiety.

An initial effort was directed to preparation of 2-hydroxyestrone 2-glucuronoside-¹⁴C 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-¹⁴C afforded the desired 2-hydroxyestrone 2-glucuronoside-¹⁴C in a satisfactory yield.

Fresh liver from male Wistar rats (*ca.* 250 g) was homogenized in ice-cooled 0.25M 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-¹⁴C (0.73 μCi, 8.3 mg), [³H-methyl]-S-adenosylmethionine (2.5 μCi), magnesium sulfate (4 mg), D-glucaro-1,4-lactone (1 mg), and 0.1M 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. Fujinori, 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)uronate³⁾ (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-³H Ether 2-Glucuronoside-¹⁴C 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-³H Ether derived from Its 2-Glucuronoside-¹⁴C 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 ¹⁴C 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. Namba, *Chem. Pharm. Bull.* (Tokyo), **22**, 687 (1974).

inherent conjugate. It has already been demonstrated that O-methylation of 2-hydroxy-estrone 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).