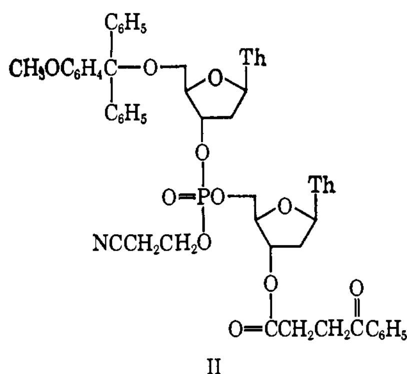
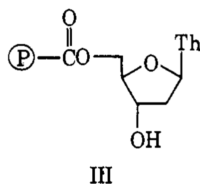
synthesis of oligonucleotides with 3′–5′ phosphodiester links.



II

With the aid of the benzoylpropionyl blocking group an improved procedure was developed for synthesis of oligothymidylate derivatives on insoluble polymer supports.[6] Thymidine joined through the 5′-oxygen to an insoluble popcorn polystyrene polymer (III) was treated successively with an excess of each of the following reagents in pyridine: (1) pyridinium β-cyanoethyl phosphate and mesitylenesulfonyl chloride, (2) 3′-O-benzoylpropionylthymidine and 2,4,6-triisopropylbenzenesulfonyl chloride,[7] and (3) 0.4 *M* hydrazine (in the pyridine–acetic acid mixture). At each stage the polymer was separated from solvent and reagents by centrifugation and washed thoroughly. Repetition of steps 1–3 followed by cleavage of the oligonucleotide products from the support with sodium hydroxide in dioxane–water yielded thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine (TpTpT), which was purified by chromatography on



III

DEAE-cellulose. The over-all yield based on thymidine bound to the support was 78%, an average of 95% for each of the chemical steps involved. Since some mechanical losses in handling the polymer undoubtedly occurred, the actual yield must have been higher. The product was completely degraded by snake venom phosphodiesterase.

(6) R. L. Letsinger and V. Mahadevan, *J. Am. Chem. Soc.*, **87**, 3526 (1965); **88**, 5319 (1966); R. L. Letsinger, M. H. Caruthers, and D. M. Jerina, *Biochemistry*, **6**, 1379 (1967).
(7) R. Lohrman and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 829 (1966).

R. L. Letsinger, M. H. Caruthers
P. S. Miller, K. K. Ogilvie
Department of Chemistry, Northwestern University
Evanston, Illinois
Received October 30, 1967

Studies on the Directive O-Methylation of Catechol Estrogens

Sir:

2-Hydroxyestrone, the principal metabolite of the estrogenic hormone estradiol, is converted *in vivo* to the 2-methyl ether without any evidence for formation of the 3-methoxy compound.[1,2] This is particularly significant because the two phenolic groups have virtually indistinguishable chemistry. Methylation *in vivo* of the important catecholamines leads nearly exclusively to the *m*-methyl ether.[3] In sharp contrast with these results in the living animal, *in vitro* methylation of catechols by rat liver O-methyl transferase enzyme is relatively indiscriminate and yields mixtures of monomethyl derivatives, often in approximately equivalent amounts.[1,4,5] The present study was undertaken to explore the possibility that sulfate conjugation, as a prior reaction stage, might be involved in the high selectivity of *in vivo* methylation.

The lack of selectivity for methylation of estrogen catechols *in vitro* was first confirmed by incubation of 200 μg of 2-hydroxyestradiol with a rat liver homogenate. 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* and the supernatant was used for the studies. The incubation medium contained 5 ml of homogenate, 1 ml of 0.1 *M* phosphate buffer, 3 mg of $MgSO_4$, and [^{14}C-methyl]-S-adenosylmethionine (2×10^5 cpm, 47.5 mCi/mmole). The mixture at pH 7.8 was shaken in air at 37° for 40 min and was then extracted with ether and washed with water, and the residue was reduced with $LiAlH_4$ to convert products oxidized at C-17 to the parent alcohols. The products were chromatographed on paper with the system formamide–cyclohexane[1] and two radioactive zones were obtained. These corresponded in mobility with reference samples of 2-hydroxyestradiol 2-methyl ether and 2-hydroxyestradiol 3-methyl ether.[6] The integrated radioactivity in the areas corresponded to 24,000 and 22,000 cpm, respectively, a product ratio of 1.1:1. The same incubation was repeated with another homogenate. Carrier amounts of 2-hydroxyestradiol 2-methyl ether and 2-hydroxyestradiol 3-methyl ether were added to the ether extract and the products were separated by careful column chromatography on alumina, acetylation, and recrystallization to constant specific activity. The product ratio found in this study was 1.2:1. The results are in agreement with findings of Knuppen and Breuer[1] with inactive compounds.

2-Hydroxyestradiol 3-sulfate[7] was incubated with rat liver homogenate under conditions identical with those used for the free steroid. In initial experiments the ether extracts were chromatographed and the papers were scanned for radioactivity. Four areas were detected, with mobilities corresponding to 2-methoxyestradiol, 2-methoxyestrone, 2-hydroxyestradiol 3-methyl ether, and 2-hydroxyestrone 3-methyl ether. The integrated areas of radioactivity of the two 17-hydroxy prod-

(1) R. Knuppen and H. Breuer, *Z. Physiol. Chem.*, **346**, 114 (1966).
(2) Following labeled estradiol administration to man the radioactive urinary 2-methoxyestrone region isolated by countercurrent distribution or partition chromatography was 85–90% homogeneous by reverse isotope dilution with authentic 2-methoxyestrone. Reverse isotope dilution of the same region with 2-hydroxyestrone 3-methyl ether indicated the absence of the labeled 3-methyl ether (unpublished observations from this laboratory).
(3) J. Axelrod, S. Senoh, and B. Witkop, *J. Biol. Chem.*, **233**, 697 (1958).
(4) S. Senoh, J. Daly, J. Axelrod, and B. Witkop, *J. Am. Chem. Soc.*, **81**, 6240 (1959).
(5) J. W. Daly, J. Axelrod, and B. Witkop, *J. Biol. Chem.*, **235**, 1155 (1960).
(6) J. Fishman, M. Tomasz, and R. Lehman, *J. Org. Chem.*, **25**, 585 (1960).
(7) M. Miyazaki and J. Fishman, *ibid.*, in press.

ucts compared to the 17-ketones were in a ratio of 5:1. The areas of the 2-methylated compounds were approximately seven times those of the 3-methoxy derivatives. Extraction of the aqueous residue following acid hydrolysis failed to give additional quantities of any of the above compounds. These initial results indicated that little or no sulfate ester of methylated catechol was present in the ether-soluble extract, and in subsequent experiments the isolation and quantitation were simplified by reduction of the extract with $LiAlH_4$ as in the studies of the unconjugated substrate. The methylated products from 2-hydroxyestradiol 3-sulfate were studied by both quantitative paper chromatography and carrier addition as in the control studies. Both methods gave virtually identical results. With the 3-sulfate as substrate 2-methoxy-3-hydroxy steroid was obtained in a yield of almost eight times that of the 3-methoxy-2-hydroxy steroid. These *in vitro* results therefore closely approximated the *in vivo* results in man. Since sulfatase activity was demonstrably present in the liver homogenate it is reasonable to suppose that the 3-methylated catechol was derived from 2-hydroxyestradiol formed prior to methylation and the substrate thus lacked the directive influence of the sulfate ester for the methylation step.

Studies were made in similar fashion using 2-hydroxyestradiol 2-sulfate[7] and rat liver homogenate with added [^{14}C-methyl]S-adenosylmethionine. This substrate yielded about twice as much 3-methoxy-2-hydroxyestradiol as the isomeric 2-methoxyestradiol. This was interpreted as an indication that the 2-sulfate ester of the estrogen catechol was more readily cleaved by sulfatase(s) present in the homogenate than was the 3-sulfate. This would result in more unesterified catechol and thus lead to a more nearly equivalent formation of the two isomeric monomethyl ethers. This possibility was examined by incubation of the 2,3-disulfate ester[7] of 2-hydroxyestrone with rat liver homogenate and added [^{14}C-methyl]S-adenosylmethionine. In accord with expectation 2-methoxyestradiol was obtained from this substrate in almost five times the yield of the 3-methoxy-2-hydroxy isomer.

These studies were repeated with a partially "purified" preparation of rat liver O-methyl transferase.[8] When 2-hydroxyestradiol was incubated with this preparation under the same conditions used with the homogenate (except that sucrose was absent) the two isomeric monomethyl ethers were formed in virtually the same ratio as with the homogenate. In contrast, both 2-sulfate and 3-sulfate monoesters of 2-hydroxyestradiol as well as the 2,3-disulfate ester were not substrates for this preparation since no detectable radioactive methylated steroid products were obtained.

These results suggest that conjugate formation *may* be an important factor in the selectivity of methylation found in intact humans. Indeed, since sulfate participation in metabolic reactions has been demonstrated,[9] it is not too daring to speculate that hydroxylation at C-2 is a reaction on the sulfate ester of estrone and/or estradiol and that only this product is methylated. The studies reported further indicate that both a methyl transferase and a sulfatase act in apparently concerted enzymic reactions in the biotransformation of catechol substrates. The fascinating implications of this conclusion are being further explored.

Acknowledgment. This investigation was supported by a grant from the American Cancer Society and Grant CA-07304 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service. The authors thank Dr. T. F. Gallagher for his interest and assistance in this work.

(8) J. Axelrod and R. Tomchick, *J. Biol. Chem.*, **233**, 702 (1958).

(9) K. D. Roberts, R. L. VandeWiele, and S. Lieberman, *ibid.*, **236**, 2213 (1961); H. I. Calvin, R. L. VandeWiele, and S. Lieberman, *Biochemistry*, **2**, 648 (1963); H. I. Calvin and S. Lieberman, *ibid.*, **3**, 259 (1964); K. D. Roberts, L. Bandi, H. I. Calvin, W. D. Drucker, and S. Lieberman, *J. Am. Chem. Soc.*, **86**, 958 (1964); S. Emerman, J. Dancis, M. Levitz, N. Wiqvist, and E. Diczfalusy, *J. Clin. Endrocrinol.*, **25**, 640 (1965); E. E. Baulieu and F. Dray, *ibid.*, **23**, 1298 (1963); H. I. Calvin and S. Lieberman, *ibid.*, **26**, 402 (1966).

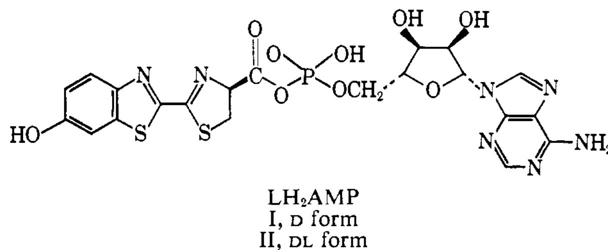
(10) (a) Visiting Fellow on leave from Chiba University, Chiba, Japan; (b) Visiting Fellow from Hokkaido University, Hokkaido, Japan.

Jack Fishman, Motoichi Miyazaki,[10a] Itsuo Yoshizawa[10b]
Institute for Steroid Research
Montefiore Hospital and Medical Center
New York, New York 10467
Received November 3, 1967

The Chemiluminescence of Firefly Luciferin. A Model for the Bioluminescent Reaction and Identification of the Product Excited State[1,2]

Sir:

Firefly bioluminescence involves the enzyme-catalyzed oxidation of luciferyl adenylate, the mixed carboxylic–phosphoric anhydride (I) of D-firefly luciferin[3] and adenosine monophosphate.[4] The stoichiometry is 1:1 with oxygen, and hydrogen peroxide is not a product.[5] With luciferase from the firefly species *Photinus pyralis*, the quantum yield of the normal yellow-green bioluminescence ($\bar{\nu}_{max}$ 17,700 cm^{-1}; FWHM[6] 2400 cm^{-1} is 0.88 ± 0.25.[7]



LH$_2$AMP
I, D form
II, DL form

The quantum yield of the red bioluminescence ($\bar{\nu}_{max}$ 16,150 cm^{-1}; FWHM 1700 cm^{-1}) observed under acidic conditions is 0.33.[8] Attempts to isolate or identify the product of the enzymatic reaction using radioactive substrates and spectroscopic techniques have

(1) Presented in part at the 154th National Meeting of the American Chemical Society, Chicago, Ill., Sept 10–15, 1967, Abstract C-027.

(2) This work was supported under Contract AT (30-1)-2802, Division of Biology and Medicine, U. S. Atomic Energy Commission, and by Public Health Service Grant No. NBO-7868 (Institute of Neurological Diseases and Blindness).

(3) E. H. White, F. McCapra, F. G. Field, and W. D. McElroy, *J. Am. Chem. Soc.*, **83**, 2402 (1961); E. H. White, F. McCapra, and G. F. Field, *ibid.*, **85**, 337 (1963).

(4) W. C. Rhodes and W. D. McElroy, *J. Biol. Chem.*, **233**, 1528 (1958); H. H. Seliger, W. D. McElroy, E. H. White, and G. F. Field, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1129 (1961).

(5) W. D. McElroy and H. H. Seliger, *Federation Proc.*, **21**, 1006 (1962).

(6) FWHM is the full band width between half-maximum intensity points of the spectrum.

(7) H. H. Seliger and W. D. McElroy, *Arch. Biochem. Biophys.*, **88**, 136 (1960).

(8) H. H. Seliger and R. A. Morton in "Photophysiology," Vol. III, A. C. Giese, Ed., Academic Press Inc., New York, N. Y., in press.