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.

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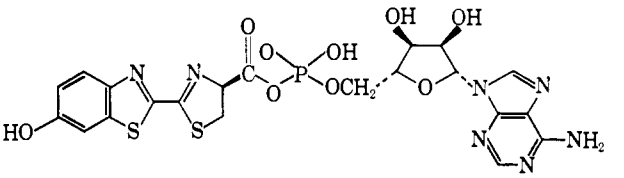
The Chemiluminescence of Firefly Luciferin. A Model for the Bioluminescent Reaction and Identification of the Product Excited State[1,2]

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_2AMP
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).

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(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).

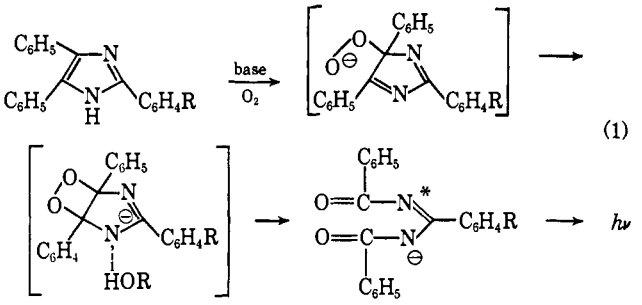
(8) H. H. Seliger and R. A. Morton in "Photophysiology," Vol. III, A. C. Giese, Ed., Academic Press Inc., New York, N. Y., in press.

failed,[9] and attention was therefore shifted to a chemiluminescent model system.[10]

We now report that the red chemiluminescence of LH_2AMP (II) in dimethyl sulfoxide (DMSO)[11] ($\bar{\nu}_{max}$ 15,900 cm^{-1}; $\bar{\nu}^{-1}_{max}$ 629 mμ; FWHM 1750 cm^{-1}) carried out under anhydrous conditions with base and oxygen has a quantum yield in excess of 0.2. Further, a red chemiluminescence of LH_2AMP ($\bar{\nu}_{max}$ 15,400 cm^{-1}; FWHM 1850 cm^{-1}) has also been observed in basic aqueous solutions containing imidazole. We consider that the three red emissions are from the same excited state in different environments. The $\bar{\nu}_{max}$ red shifts of 250 and 500 cm^{-1} and the constancy of the spectral shapes (FWHM ~1750 cm^{-1}) in going from red bioluminescence to DMSO chemiluminescence to aqueous chemiluminescence suggest a progression from less to more "polar" surroundings.[12]

We have observed a short-lived fluorescence emission of spent chemiluminescence reaction mixtures of LH_2AMP in DMSO whose spectrum is identical[13] with the chemiluminescence. The product of this chemiluminescence proved to be too unstable to isolate, however, and attention was shifted to analogs that promised to give more stable products.

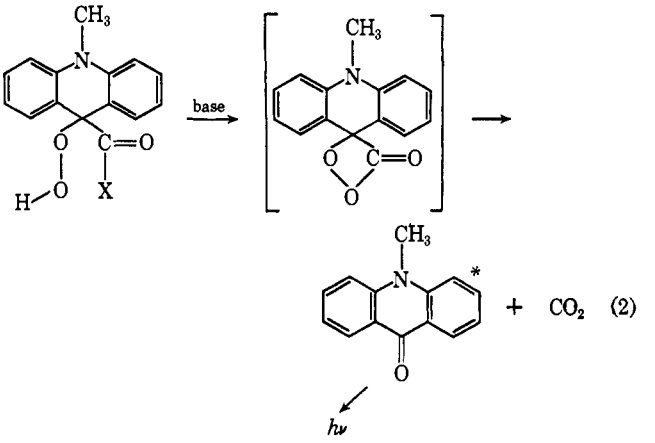
The general nature of the chemiluminescent reaction of II suggested a close relationship to the chemiluminescence of lophine derivatives[14] (eq 1) and to the chemi-



(1)

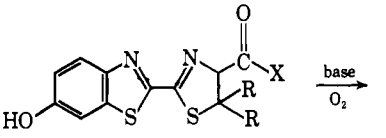
luminescence of the acridinium carboxylic acids (eq 2).[15] The analogous reactions for luciferin derivatives are given by eq 3.

Confirmation of this mechanism has come from a study of the 5,5-dimethyl derivative of luciferin (IV, X = OH);[16] the spectroscopic properties of this compound are identical with those of luciferin. Furthermore, the chemiluminescence emission spectrum of the 5,5-dimethylluciferyl adenylate (IV, X = adenylate) is identical with the chemiluminescence emission spectrum

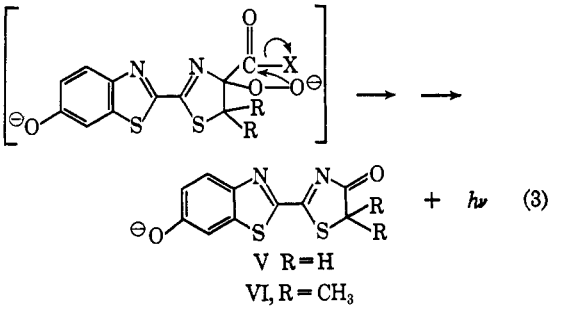


(2)

X = conjugate base of a strong acid



III, R = H
IV, R = CH_3
X, conjugate base of a strong acid



(3)

V R = H
VI, R = CH_3

of II. The proposed chemiluminescence product VI has now been synthesized by the condensation of ethyl α-mercaptoisobutyrate with 2-cyano-6-hydroxybenzothiazole.[17] The fluorescence emission spectrum of the anion VI in DMSO is identical with the chemiluminescence emission spectrum of IV and, further, to the fluorescence emission spectrum of spent chemiluminescence reaction mixtures of IV.

Further evidence for this mechanism of the light production comes from the findings that (1) compound I is readily racemized, (2) the chemiluminescence of II labeled at C-4 with deuterium proceeds more slowly than that of II, (3) for efficient chemiluminescence of III, X must be the conjugate base of a strong acid, and (4) the O-methyl ether[16] of I is not effective in either chemiluminescence or bioluminescence. We believe, therefore, that both the luciferyl adenylate red bioluminescence and the chemiluminescence emissions are from the excited state of the monoanion V.

Little evidence is available concerning the yellow-green emitting species in bioluminescence. We feel that the excited state of the dianion of V is a likely candidate. Consistent with this view is the more basic pH necessary for this emission[8] and our observation that IV

(9) The spectroscopic experiments are outlined by H. H. Seliger and W. D. McElroy in "Bioluminescence in Progress," F. H. Johnson and Y. Haneda, Ed., Princeton University Press, Princeton, N. J., 1966, p 405.

(10) The base–solvent system was first used for the chemiluminescence of luminol (E. H. White, *J. Chem. Educ.*, **34**, 275 (1957)), and applied to luciferin by H. H. Seliger and W. D. McElroy, *Science*, **138**, 683 (1962).

(11) The low-intensity greenish emission reported in addition to the red emission (ref 2 of ref 10) is most likely the weak chemiluminescence of unreacted luciferin in previous LH_2AMP preparations. This does not occur in our present preparations.

(12) E. Lippert, *Angew. Chem.*, **73**, 695 (1961); W. O. McClure and G. M. Edelman, *Biochemistry*, **5**, 1908 (1966).

(13) By "identical" we mean agreement in $\bar{\nu}_{max}$ ±0.5% (~2 mμ) and in FWHM ±3%.

(14) E. H. White and M. J. C. Harding, *J. Am. Chem. Soc.*, **86**, 5686 (1964); E. H. White and M. J. C. Harding, *Photochem. Photobiol.*, **4**, 1129 (1965).

(15) F. McCapra and D. G. Richardson, *Tetrahedron Letters*, 3167 (1964).

(16) E. H. White, H. Worther, G. F. Field, and W. D. McElroy, *J. Org. Chem.*, **30**, 2344 (1965).

(17) The structure of the product (phenolic form of VI) follows from its elemental analyses and its infrared and nmr spectra.

(X = adenylate) is inactive in enzymatic light production.

(18) National Institutes of Health Predoctoral Fellow, Grant No. GM-57.

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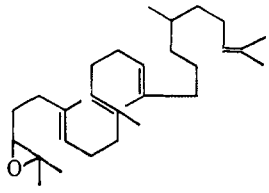
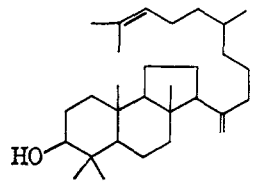
Enzymic Cyclization of *trans,trans,trans*-18,19-Dihydrosqualene 2,3-Oxide

Sir:

With the hope of shedding light on the cyclization stage of lanosterol biosynthesis, we have carried out enzymic experiments on an appropriately modified substrate, [4-^{3}H]*trans,trans,trans*-18,19-dihydrosqualene 2,3-oxide (I).[1,2] We find that the rat liver enzyme preparation which effects the normal biosynthesis of



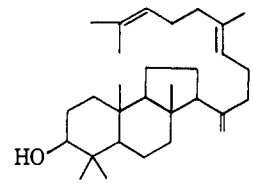
I



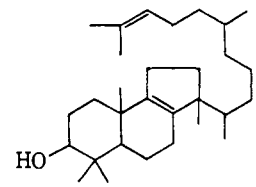
II(A,B)

lanosterol from squalene 2,3-oxide also converts in reasonable yield this dihydrosqualene oxide to the perhydrocyclopenta[*a*]naphthalene derivative A (gross structure II), representing a skeletal type previously encountered as a nonenzymic product (III) of squalene oxide cyclization.[4]

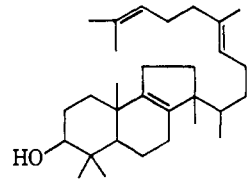
Enzymic reaction of the radiolabeled 18,19-dihydrosqualene 2,3-oxide (I) was carried out in a clarified (110,000*g* supernatant) preparation of 2,3-oxidosqualene–lanosterol cyclase isolated from the microsomal fraction of rat liver.[5] In an exemplary run, 1.179 mg of oxide (60.00×10^6 dpm; specific activity = 51,300 dpm/μg) was incubated for 4 hr at 37° under nitrogen with 65 ml of enzyme preparation equivalent to 45 g of rat liver. The radioactive material (54.76×10^6 dpm), following saponification and extraction, was separated by tlc[7] to yield as a major product (~8% yield) a substance A (R_f 0.41; lanosterol gave R_f 0.37). By these means, we accumulated a total of 220 μg of product A on which the experiments described below were carried out. For comparison purposes, nonenzymic cyclization products B (gross structure II) and C and D (gross structure IV) were prepared from 18,19-dihydrosqualene 2,3-oxide (I) by means previously utilized for the production of the analogous tricycles III[4] and V [(C′)[4] and (D′)[8]] from squalene 2,3-oxide.



III



IV (C, D, E, F)



V (C′, D′)

By means of a 100-Mc Varian instrument, there was obtained a time-averaged nmr spectrum of enzymic product A (220 μg in CCl_4) which displayed the following peaks (values relative to TMS = 10.0): 4.92 (1 H, triplet), >C=CH–; 5.17 (1 H, singlet) and 5.42 (1 H, singlet), >C=CH_2; 6.85 (1 H, triplet), axial >CHO(H); 8.35 (3 H, singlet) and 8.42 (3 H, singlet), two >C=C-(CH_3)–; and 9.05, 9.10, 9.16, and 9.27 (*ca.* five methyls on saturated carbon). Essentially identical resonances for comparable protons were observed in a 60-Mc nmr spectrum of III.[4]

The mass spectra of the TMSE derivatives of the tricyclic alcohols A and B display fragmentation patterns[9] which differ *only* in the relative intensity of certain peaks; *cf.* *m/e* 189, 190, 191 [$C_{14}H_{21-23}$], and 229 [$C_{17}H_{25}$] which are characteristic of this carbon skeleton and consistent with gross structure II and are in accordance with analogous assignments and conclusions in the squalene series (structure III). Although possessing identical R_f values (0.41) on tlc, substances A (glpc, R_c = 1.60) and B (glpc, R_c = 1.91) exhibit glpc retention times in the ratio 0.83:1.[10] Certain relative peak intensity differences in these otherwise identical fragmentation patterns and their glpc behavior indicate structural formulation of compounds A and B as stereoisomers.

In order to confirm structural assignment II to sub-

(1) Synthesis: E. E. van Tamelen, K. B. Sharpless, and R. Hanzlik, *J. Am. Chem. Soc.*, in press.

(2) Enzymic conversion of squalene and squalene 2,3-oxide variants (22,23-dihydro and 23,24,24′-trisnor) to lanosterol-like systems was first disclosed by E. E. van Tamelen, K. B. Sharpless, J. D. Willett, R. B. Clayton, and A. L. Burlingame, *ibid.*, **89**, 3920 (1967). The 22,23-dihydrosqualene oxide case was duplicated by E. J. Corey and S. K. Gross, *J. Am. Chem. Soc.*, **89**, 4561 (1967), who reported in addition the enzymic transformation of squalene 2,3:22,23-dioxide[3] to lanosterol 24,25-oxide.

(3) For preparation, see E. E. van Tamelen and T. J. Curphey, *Tetrahedron Letters*, 121 (1962).

(4) E. E. van Tamelen, J. D. Willett, M. Schwartz, and R. Nadeau, *J. Am. Chem. Soc.*, **88**, 5937 (1966).

(5) The method used for this preparation was a modification of that indicated by Dean, *et al.*[6] Its properties will be described elsewhere.

(6) P. D. G. Dean, P. R. Ortiz de Montellano, K. Bloch, and E. J. Corey, *J. Biol. Chem.*, **242**, 3014 (1967).

(7) All thin layer R_1 values refer to mobilities on unactivated silica gel G plates which were eluted with a solution of 20% ethyl acetate in hexane.

(8) D′ (V) is a new tricyclic alcohol which was recently isolated from the chemical cyclization which yields the known tricycle C′ (V). The mass spectra and nmr spectra of D′ and of its 18,19-dihydro analog D indicate that these compounds are stereoisomeric with the known C′ and C structures, respectively (unpublished results, K. B. Sharpless).

(9) These were determined under identical conditions assuring quantitatively reproducible fragmentation patterns using an A.E.I. MS-12 mass spectrometer.

(10) Glpc data were obtained for trimethylsilyl ether on terminated Carbowax (5%) ("steroid analytical phase," Wilkins Instrument Co.) on Chromosorb W at 218° with a nitrogen flow rate of 90 cc/min; R_c = retention time relative to cholestane.