The structure was solved by Patterson and Fourier methods and refined by least squares to an R of 0.058. The ethoxy group is disordered, but as yet the nature of this disorder is not understood. The positions of the five hydrogen atoms of the bis(thiosemicarbazone) chains have been located in a difference map but have not yet been included in the calculations.

In the crystal structure of I the chain from S(5) to S(5′) is fully extended, and the conjugated system from N(3) to N(3′) (excluding the side-chain atoms) is approximately planar. The hydrogen atoms on N(3) and N(3′) of I are ionized on chelate formation to give II, where one molecule of I is coordinated to one cupric ion as a tetradentate chelate. The coordination is *via* the nitrogen atoms N(2) and N(2′) and the two sulfur atoms, which are at the corners of a very distorted square.

All the atoms of II [except for hydrogen atoms and those of the side chain on C(1′)] lie very close to the same plane, the maximum deviation from the least-squares best plane through Cu, N(6), C(4), N(3), N(2), C(1), C(1′), N(2′), N(3′), C(4′), N(6′) being about 0.06 A. The two sulfur atoms lie one above (+0.33 A) and one below (−0.11 A) this plane. These deviations are directed toward the copper atoms of adjacent complex molecules, which pack with their planes parallel to the reference molecule. Therefore there are sulfur atoms from neighboring molecules on both sides of the planar complex, completing a sixfold coordination for the copper atom. Although these Cu···S packing distances of 3.10 and 3.31 A are considerably longer than single bonds, there appears to be a real tendency toward bond formation since the Cu···S directions are very close to normal to the plane of the complex. Owing to repulsion between other parts of the molecules these Cu···S distances cannot be any shorter.

It is possible that in solution one or both of these octahedral positions are strong binding sites for a ligand that is not sterically hindered by the remainder of the atoms of II. This type of binding could bring I into favorable stereochemical relationships with other reacting species and thereby enhance its biological activity.

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Steroid Hydroxylations. III. The Role of Reduced Nicotinamide–Adenine Dinucleotide Phosphate (NADPH)

Sir:

Proof of the exact mechanism of steroid hydroxylations in biological systems has not been demonstrated. Hayano and co-workers showed that the oxygen atom incorporated during adrenal C-11 hydroxylation came from molecular oxygen rather than from water.[1] Subsequent experiments have shown that the same holds true for other positions on the steroid nucleus.[2]

To date, reduced pyridine coenzymes and adenosine 5-triphosphate (ATP) have been shown to be necessary for hydroxylations.[3] Stoichiometric experiments by Cooper, *et al.*, performed during C-21 hydroxylation show that a 1:1 molar relationship exists between O_2 and NADPH.[4] Several different steroid hydroxylation mechanisms, all involving essentially the hydroxonium ion (OH^+), have been proposed by Hayano.[5] The hydroxylation reaction of progesterone to yield 17-hydroxyprogesterone was chosen to demonstrate the role of NADPH.

Glucose-1-^{3}H 6-phosphate was prepared by the phosphorylation of D-glucose-1-^{3}H (New England Nuclear Corp.).[6] Subsequent reaction of the glucose-1-^{3}H 6-phosphate with NADP and glucose 6-phosphate dehydrogenase gave the reduced product (340 mμ absorbancy), which was separated chromatographically according to the procedure of Pastore and Friedkin.[7] The microsomal fraction of sow ovaries was isolated[8] and the particles were checked for homogeneity by electron microscopy. Incubations, in duplicate, of 1 mg of progesterone (Calbiochem) with added NADPH (expt 1, 803,460 dpm, specific activity 6950 dpm/μg; expt 2, 1,606,920 dpm, specific activity 13,910 dpm/μg) were performed as previously described.[8] A Packard TriCarb liquid scintillation spectrometer, Model 314EX-2, was used for measurement of radioactivity. The steroids were dissolved in 10 ml of scintillation mixture prepared by dissolving 1 g of PPO + 0.06 g of POPOP in 200 ml of toluene. A Vanguard Model 880-D paper strip autoscanner was used for location of radioactive compounds on paper. Zaffaroni paper chromatographic systems with formamide as the stationary phase were utilized throughout this investigation.

After incubation for 1.5 hr at 37° the reaction was stopped with four volumes of acetone. Storage in a cold room overnight completed the precipitation of proteins, which were removed by filtration. The acetone was carefully removed *in vacuo*, and the resulting water layer was extracted three times with equal volumes of chloroform. The water layer was subjected to a distillation, and the distillate was found to contain a high amount of radioactivity (expt 1, 600,000 dpm, and expt 2, 1,050,000 dpm). The residue from the distillation, and the protein precipitates, were dissolved in 0.5 ml of solubilizing solution (Nuclear-Chicago Corp.) and measured for radioactivity. No significant counts over background could be detected.

The chloroform extract, after addition of authentic 17-hydroxyprogesterone (300 μg), was chromatographed in a hexane system for 20 hr and then further developed in a hexane–benzene system. After elution of this area with methanol and rechromatography in a benzene system, structure proof was undertaken to determine where the tritium label was located on the steroid nucleus. This was accomplished by using one portion from each experiment for C-20 ketone reduction with sodium borohydride followed by periodic acid cleavage

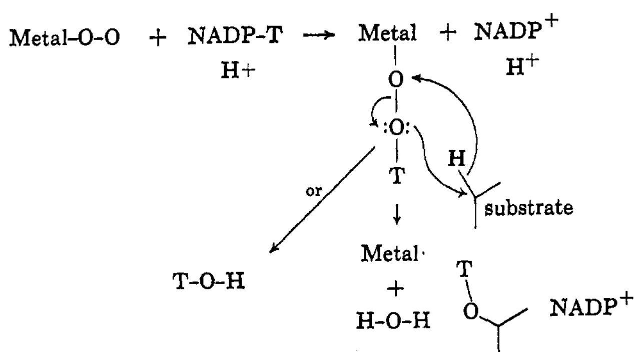
(1) M. Hayano, M. C. Lindberg, R. I. Dorfman, J. E. H. Hancock, and W. von E. Doering, *Arch. Biochem. Biophys.*, **59**, 529 (1955).
(2) P. Talalay, *Ann. Rev. Biochem.*, **34**, 347 (1965).
(3) J. E. Plager and L. T. Samuels, *J. Biol. Chem.*, **211**, 21 (1954).
(4) D. Y. Cooper, R. E. Estabrook, and O. Rosenthal, *ibid.*, **238**, 1320 (1963).
(5) M. Hayano in "Oxygenases," O. Hayaishi, Ed., Academic Press Inc., New York, N. Y., 1962, pp 181–240.
(6) J. M. Lowenstein, *Methods Enzymol.*, **6**, 877 (1963).
(7) E. J. Pastore and M. Friedkin, *J. Biol. Chem.*, **236**, 2314 (1961).
(8) J. H. Ball and B. Kadis, *Arch. Biochem. Biophys.*, **110**, 427 (1965).

Table I. Structure Proof of Isolated 17-Hydroxyprogesterone

Compd and deriv formation	Chromatography system (R_f)	Expt 1,[a] dpm Steroid	Expt 1,[a] dpm Water	Expt 2,[a] dpm Steroid	Expt 2,[a] dpm Water
17-Hydroxyprogesterone (262 μg isolated)	Hexane–benzene (0.23)	87,940 (340)	. . .	171,100 (1050)	. . .
(248 μg isolated)	Benzene (0.64)	87,735 (339)	. . .	170,050 (1047)	. . .
$NaBH_4$ reduction (20α-hydroxypregn-4-en-3-one)	Benzene (0.29)	30,330 (337)	b	84,780 (1048)	
Periodic acid oxidation (androst-4-ene-3,17-dione)	Hexane–benzene (0.76)	b	24,760	b	75,680
Acetylation (17-acetoxyprogesterone)	Hexane (0.1)	b	26,500	b	77,520

[a] Values in parentheses represent specific activity, dpm/μg. [b] No significant counts over background.

of the glycol. If the tritium label was on the hydrogen of the 17-hydroxyl group, the reduced compound would still be radioactive, while the cleaved product would not. Another portion of the tritium-labeled 17-hydroxyprogesterone was acetylated according to the procedure of Turner.[9] Again, if the label was on



the hydrogen atom of the 17-hydroxyl group, no activity should be detected in the acetylated derivative. Table I shows the results of these experiments. The compounds, once eluted from the paper, were partitioned between water and chloroform to remove absorbing material. Steroid concentrations were then determined with a Beckman Model DU spectrophotometer by comparing the 240-mμ absorption peak with known quantities of material.

(9) R. B. Turner, *J. Am. Chem. Soc.*, 75, 3489 (1953).

The results of these experiments are consistent with the mechanism portrayed by Hayano[5] since the tritium label from the NADPH was found in both the water fraction and the isolated 17-hydroxyprogesterone. This mechanism can be pictured as occurring at a single active site where the oxygen, NADPH, and substrate are brought together on the enzyme surface. No exchange of tritium between NADPH and water occurs.[10] Other radioactive materials were detected, and it is hoped that their structures can be determined.

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(10) Personal communication from Dr. M. Friedkin.

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Book Reviews

Oxygen. Elementary Forms and Hydrogen Peroxide. By Michael Ardon, Hebrew University of Jerusalem. W. A. Benjamin, Inc., 1 Park Ave., New York, N. Y. 1965. x + 106 pp. 15.5 × 23.5 cm. $6.75.

This little book belongs to the "Physical Inorganic Chemistry Series" launched a couple of years ago by Sienko and Plane. Thus the inorganic chemists, like their organic *confrères*, acknowledge their dependence on the modern methods of physics. As implied by the title, this is a descriptive sort of monograph on oxygen, the element, and one of its innumerable compounds, hydrogen peroxide. The author warns in his preface that he did not intend a nutshell presentation of this wide field, nor a miniature reference book. His endeavor seems justified by the fact that recent monographs on oxygen have appeared as part of large treatises either in German (Gmelin's, 8th ed, 1963–1964) or in French (Pascal, "Nouveau Traité de Chimie minérale," Tome XIII, 1960).

The first chapter, on the element and its atom, deals with such topics as the separation of the isotopes of oxygen, so successfully realized in Israel, the electronic structure, and chemical bonding. The second chapter contains a feature, too often neglected in modern textbooks on inorganic chemistry, namely, a detailed discussion