EPOXIDATION OF UNSATURATED STEROIDS BY MICROÖRGANISMS[1]

Sir:

Fermentation studies with certain steroidal substrates have revealed *a new type of bio-oxygenative transformation, the stereospecific epoxidation of isolated unsaturation in steroids.* While incubating a group of unsaturated derivatives of Reichstein's Compound S with microörganisms of familiar hydroxylating capacity, we have observed that enzymatic epoxidation of non-conjugated unsaturation occurs concomitantly with previously recognized hydroxylation processes at saturated centers.[2] Subsequent experiments with a limited number of different microörganisms strongly suggest that the occurrence of this type of transformation is not a random phenomenon, but rather a predictable one.

When $\Delta^{4,9(11)}$-pregnadiene-17α,21-diol-3,20-dione (I)[3] was bio-oxygenated with *Curvularia lunata* (N.R.R.L. 2380)[4] or *Cunninghamella blakesleeana* (A.T.C.C. 9245),[5] the presence among the products of Δ^4-9β,11β-epoxidopregnene-17α,21-diol-3,20-dione (II), m.p. 210–211°; $[\alpha]_D$ $+13°$ (dioxane); λ_{max}^{EtOH} 244 mμ ($\log \epsilon = 4.17$); λ_{max}^{KBr} 2.92, 5.87, 6.10, 6.23; (*Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 70.0; H, 7.83. Found: C, 69.7; H, 7.60),[6] could be demonstrated by conventional paper chromatographic techniques. In evaluating the *C. lunata* fermentation using a modified Bush[7] chromatographic system (5:1 toluene–ethanol developing phase on paper impregnated with 7:3 acetone–water), the presence of a component migrating exactly like authentic II could be demonstrated ($R_f = 0.57$; cortisone $R_f = 0.40$). This component, which displayed the expected ultraviolet absorption and reduced "blue tetrazolium," migrated after acetylation exactly like the 21-monoacetate of II[6] in any of a variety of systems (*e.g.*, benzene–formamide, $R_f = 0.47$; cortisone $R_f = 0.28$). Elution of the appropriate zone with chloroform afforded crystalline epoxide whose infrared spectrum proved to be identical with that of an authentic sample of Δ^4-9β,11β-epoxidopregnene-17α,21-diol-3,20-dione 21-acetate, λ_{max}^{KBr} 2.95, 5.81 (shoulder), 5.88, 6.08, 6.23, 8.18.[6] Other compounds formed in this complex fermentation included further transformation products of both I and II.[8]

The occurrence of microbiological epoxidation upon incubation of an unsaturated steroidal substrate with an appropriate microörganism was also demonstrable with a variety of known 14α-hydroxylating cultures. When $\Delta^{4,14}$-pregnadiene-17α,21-diol-3,20-dione (III),[9] m.p. 196.8–198.8°; $[\alpha]_D$ $+52°$ (dioxane); λ_{max}^{EtOH} 240 mμ ($\log \epsilon = 4.21$); λ_{max}^{KBr} 2.98, 5.85, 6.04, 6.22; (*Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.2; H, 8.19. Found: C, 72.9; H, 7.97), was incubated with either *Curvularia lunata*,[4] *Cunninghamella blakesleeana*,[5] *Helicostylum piriforme* (A.T.C.C. 8992 and 8686),[10] *Mucor griseocyanus* (A.T.C.C. 1207a)[10] or *Mucor parasiticus* (A.T.C.C. 6476),[10] the corresponding epoxide, Δ^4-14α,15α-epoxidopregnene-17α,21-diol-3,20-dione (IV),[9] m.p. 229.6–232.2°; $[\alpha]_D$ $+135°$ (dioxane); λ_{max}^{EtOH} 239 mμ ($\log \epsilon = 4.24$); λ_{max}^{KBr} 2.94, 5.83, 6.09, 6.23; (*Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 70.0; H, 7.83. Found: C, 69.7; H, 7.50), invariably was formed. Microörganisms incapable of causing the 14α-hydroxylation of typical substrates such as Compound S, including *Rhizopus nigricans* (A.T.C.C. 6227b),[11] *Rhizopus arrhizus* (A.T.C.C. 11145)[11] and *Pestalotia foedans* (A.T.C.C. 11817),[12] were similarly unable to effect the epoxidation of III. Detection of the epoxide IV in mixtures of fermentation products was facilitated by its unusual migration rate. In both Zaffaroni and Bush paper chromatographic systems this compound is markedly less polar than Compound S (*e.g.*, 1:1 benzene–chloroform/formamide IV, $R_f = 0.74$; Compound S, $R_f = 0.50$)). In two instances (*C. lunata* and *H. piriforme*) the epoxidic transformation product was isolated by partition chromatography over silica gel and shown to be identical with a sample of IV prepared chemically[9] by comparison of the infrared spectra.

The results of these preliminary experiments on microbiological epoxidation have proven amenable to correlation. In each of the several cases studied, *a microörganism capable of introducing an axial hydroxyl function at C_n of a saturated steroid also effected the introduction of an epoxide grouping "axial" at C_n in the corresponding unsaturated substrate.*

In order to ascertain whether equatorial-hydroxylating cultures could also effect the epoxidation of appropriate substrates, I was incubated with 11α-hydroxylating cultures such as *Rhizopus nigricans*, *Rhizopus arrhizus* and *Pestalotia foedans*. Formation of the corresponding epoxide, Δ^4-9α,11α-

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[1] Paper II in the series Microbiological Conversion of Steroids; for previous paper, see G. M. Shull and D. A. Kita, THIS JOURNAL, **77**, 763 (1955).

[2] In order to establish these epoxidations as *bona fide* enzymatic reactions and eliminate the possibility that air oxidation of substrate was occurring, control experiments were carried out with tissue which had previously been inactivated by autoclaving or by treatment with 0.01 *M* sodium azide. Under these conditions epoxidation of unsaturated substrates was not observed, thereby demonstrating that the process was enzymatic in nature.

[3] S. Bernstein, R. Littell and J. H. Williams, THIS JOURNAL, **75**, 4830 (1953).

[4] G. M. Shull and D. A. Kita, *ibid.*, **77**, 763 (1955); G. M. Shull, D. A. Kita and J. W. Davisson, U. S. Patent 2,658,023 (November 3, 1953).

[5] F. R. Hanson, *et al.*, THIS JOURNAL, **75**, 5369 (1953); K. M. Mann, F. R. Hanson and P. W. O'Connell, *Fed. Proc.*, **14**, 251 (1955).

[6] The two isomeric 9,11-epoxides derived from Compound S acetate have been described by J. Fried and E. F. Sabo, THIS JOURNAL, **75**, 2273 (1953).

[7] I. E. Bush, *Biochem. J.*, **50**, 370 (1952).

[8] The isolation and identification of these other transformation products will be the subject of a more detailed communication from this laboratory.

[9] B. M. Bloom, E. J. Agnello and G. D. Laubach, *Experientia*, in press (1955).

[10] P. D. Meister, *et al.*, Abstracts of Papers, 123rd American Chemical Society Meeting, Los Angeles, California, March 15–19, 1953, p. 5C; H. C. Murray and D. H. Peterson, U. S. Patent 2,602,769 (July 8, 1952), U. S. Patent 2,673,866 (March 30, 1954).

[11] S. H. Eppstein, *et al.*, THIS JOURNAL, **75**, 408 (1953); D. H. Peterson, *et al.*, *ibid.*, **75**, 412 (1953); P. D. Meister, *et al.*, *ibid.*, **75**, 416 (1953).

[12] G. M. Shull, J. L. Sardinas and J. B. Routien, Canadian Patent 507,009 (November 2, 1954).

epoxidopregnene-17α,21-diol-3,20-dione,[6] was not observed in these fermentations.[13,14]

These studies would seem to preclude I as an intermediate in the microbiological synthesis of hydrocortisone from Compound S. In this respect they complement the findings of Hayano and Dorfman[15] with mammalian adrenal enzyme systems.

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[13] In another experiment $\Delta^{4,16}$-pregnadiene-3,20-dione was incubated with *Actinomycete* A.T.C.C. 11009, a 16α-hydroxylating organism (D. Perlman, E. Titus and J. Fried. THIS JOURNAL, **74**, 2126 (1952)). The formation of Δ^4-16α,17α-epoxidopregnene-3,20-dione could not be demonstrated.

[14] Extension of these studies to include other types of microörganisms and additional substrates is in progress. A discussion of the mechanistic implications of microbiological epoxidation will appear elsewhere.

[15] M. Hayano and R. I. Dorfman, *J. Biol. Chem.*, **211**, 227 (1954). Rather contrary findings are described by K. Miescher, A. Wettstein and F. W. Kahnt, *Acta Physiologica Latino Americano*, **3**, 144 (1953).

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BOOK REVIEWS

An Advanced Treatise on Physical Chemistry. Volume V. Molecular Spectra and Structure. Dielectrics and Dipole Moments. By J. R. PARTINGTON, M.B.E., D.Sc., Emeritus Professor in the University of London. Longmans, Green and Co., 55 Fifth Avenue, New York 3, N. Y. 1955. x + 565 pp. 17 × 25.5 cm. Price, $15.50.

The present volume of Professor Partington's treatise is concerned with molecular spectra, dielectric constants and dipole moments. Inclusion of these topics in one volume is appropriate, and results in a comprehensive survey of two important aspects of molecular structure. The treatise should be particularly valuable to the graduate student in physical chemistry who is preparing for examinations. At the same time it is a useful reference work for specialists. The style of writing is exceptionally clear, and should appeal both to specialist and non-specialist.

The form of presentation is unusual, and may be described as a mixture of experimental description, physical theory and pure mathematics. If the last component appears to be overemphasized, it must be admitted that the mathematical background is unusually complete for a text of this kind. Consequently there is less need for consultation of other texts. For example, applications of quantum mechanical theory to problems of molecular structure is accompanied by an unusually complete (60 page) treatment of spherical harmonics, and later by a 32 page discussion of symmetry and group theory. Many painful details are included which are frequently avoided by other writers, or relegated to appendices or footnotes. Similarly, the treatment of dielectrics begins with a full discussion of elementary theory of alternating current circuits. Mention should be made also of the large number of literature references (4056, according to the author) in the present volume. This feature should be particularly helpful to the serious student.

The discussion of experimental material is uneven. It is rather brief in connection with molecular spectra, but rather complete in connection with dielectric phenomena. Methods of dielectric measurements are described in some detail, and are well illustrated by numerous diagrams of apparatus and electrical circuits. Full tables are given of dielectric constants, Kerr effect data and of dipole moments. The relation of dipole moments to molecular structure is discussed fully.

The treatise is less satisfactory in some aspects of fundamental physical theory, particularly the theory of molecular spectra. Discussion of the origins and meaning of infrared and electronic spectra are brief, and perhaps inadequate. The "simple" theory of the Raman effect on page 28 is certainly unsatisfactory, although a more nearly correct discussion is given later on pages 48 and 54. It appears that a more straightforward explanation of molecular orbitals might have been given and more extensive discussion based on recent calculations might have been included.

This volume will certainly not appeal to hurried readers who seek a streamlined account of molecular structure. Others may reject it for aesthetic reasons, for when experimental details, physical theory and all necessary mathematics are included in juxtaposition, some sense of logical development inevitably will be lost. This feature should not detract, however from its utility as a valuable reference work for the serious student of molecular structure.

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Biochemistry of Nitrogen. Series A. II. Chemica Nr 60 A Collection of Papers on Biochemistry of Nitrogen and Related Subjects. Dedicated to Artturi Ilmari Virtanen. Editorial Board: N. J. TOIVONEN, E. TOMMILA, J. ERKAMA, P. ROINE, J. K. MIETTINEN. Suomalainen Tiedeakatemia, Publishers, Helsinki, Finland. 1955. 535 pp. 17.5 × 25 cm. Price, 2300 Mk., Bound 2800 Mk.

"The contributions of this volume offer an international tribute to an esteemed scientist" . . . . "who has laid the foundation for Finnish biochemistry."

The volume consists of forty-eight papers each of which bears a relationship to the past work of Virtanen. This evidence of intellectual ancestry is not surprising in view of the extensive investigative activity of the honoree and his profound influence upon knowledge in this field. Most of the papers report experiments in an amount of detail which characterizes journal articles. Some of the papers, however, are brief reviews. These latter include, "The Synthesis of Nucleotide Coenzymes," by A. R. Todd; "Nitrogen-Deficient Microörganisms: A New Technique in Microbiological Chemistry," by J. De Ley; "Mutations and Adaptations in Bacteria," by C. N. Hinshelwood; "Étude de l'action de quelques cations divalents sur les combinaisons acide phytique-protéine," by R. Barré, J. E. Courtois, and G. Wormser; "Application of the Symbiosis Phenomenon among Lactic Acid Bacteria to the Study of the Biosynthetic Pathways of Growth Factors," by Veikko Nurmikko; "Activity-*p*H-curve of Yeast Invertase and the Mechanism of Hydrolase Action," by Karl Myrbäck; "Gedanken über die Bedeutung der makromolekularen Chemie für die Biologie," by Hermann and Magda Staudinger; "Chromatographic Experiments with Proteins," by Arne Tiselius; "Recent Advances in the Chemistry of Some Natural Polymers Containing Amino Sugars," by M. Stacey; "Antibiotics and Nitrogen Excretion—With Special Reference to Penicillin," by W. H. Peterson; "Nitrogenous Compounds in Plants: Recent Knowledge Derived from Paper Partition Chromatography," by F. C. Steward, R. M. Zacharius, and J. K. Pollard; "The Energy Change in Organic Rearrangements and the Electronegativity Scale," by Linus Pauling; "Remarks on the Physiological Importance of the Nucleoside Phosphotransferases," by Erwin Chargaff; and "A Universal Definition of the Concept of ≫Buffer≫, Founded on Brønsted's Acid-Base Definition," by Holger Jørgensen.

Representative articles describing primarily sets of experiments include, "Photoperiodism and Photosynthetic