medium made of equal volumes of 0.2 *M* phosphate buffer *p*H 7.2 and 1.15% KCl. The homogenate was centrifuged in the Spinco ultracentrifuge at 90,000 × *g* for 30 minutes. The enzyme system contained in the supernatant fluid was salted out by the careful addition of solid ammonium sulfate at 0° (56 g. per 100 ml. initial volume) and was redissolved in 0.05 *M* phosphate buffer of *p*H 7.2. The assay for enzymatic activity was carried out in test tubes by incubating an aliquot of the enzyme solution with inositol. At the end of the incubation the tubes were heated for 2 minutes at 100° and the protein-free filtrate was assayed for glucuronic acid using the orcinol method of Mejbaum.[1] Table I summarizes a typical experiment. When inositol was omitted from the incubation mixture or when the enzyme solution was heat-

TABLE I

RATE OF FORMATION OF GLUCURONIC FROM INOSITOL

The reaction mixture contained 0.2 ml. of 1 *M* phosphate buffer *p*H 7.2, 0.2 ml. of 0.5 *M* inositol, 0.15 ml. of enzyme solution and distilled water to a final volume of 2 ml.; gas phase oxygen, incubation at 34°.

Time of incubation (minutes)	5	10	15	20	30	40
Glucuronic acid (γ)	50	75	101	118	147	160

inactivated prior to its use the amount of orcinol-reacting substances was 3–5% of that formed in the complete system.

In a large scale experiment in which 15 ml. of enzyme solution was used, 78.3 mg. of glucuronic acid was formed representing a 10% conversion of the added inositol. The glucuronic acid was isolated by column chromatography on Dowex-1 X10 (acetate form) using 0.4 *N* acetic acid as the eluting solvent and was identified by paper chromatography using the solvent systems: pyridine–ethyl acetate–acetic acid–water,[2] ethanol–water (88:12), acetone–water (80:20), *n*-butanol–acetic acid–water (100:21:50), and ethanol–acetic acid–water (80:10:10). The spots were revealed by spraying the paper with aniline oxalate and heating at 100° for 5 minutes. In all cases the isolated uronic acid migrated in a manner indistinguishable from authentic glucuronic acid and was resolved from guluronic, galacturonic, mannuronic, iduronic, 2-ketogluconic, 5-ketogluconic, and ascorbic acid, as well as from the corresponding lactones of the above uronic acids.

The sodium salt of the isolated uronic acid was crystallized from ethanol (*Anal.* Calcd. for $C_6H_9O_7Na \cdot H_2O$: C, 30.77: H, 4.70. Found: C, 30.86; H, 4.74). The free acid has a m.p. of 162–163° while the lactone had a m.p. of 176–178°. The brucine salt of the isolated uronic acid had a m.p. of 162–165°.

The isolated glucuronic acid as well as its lactone were optically inactive, indicating that it is a racemic mixture. *Escherichia coli* (Strain B) adapted on D-glucuronic acid utilized approximately half of a known amount of the isolated uronic acid. The remaining half was recovered from the culture medium and its specific rotation at equilibrium was found to be $[\alpha]^{24}_D$ −33.3° (*c* 0.48 in H_2O, *1 l* dm.) compared to −36.0° expected for L-glucuronic acid. The enzymatic formation of glucuronic acid from inositol occurs with the simultaneous uptake of oxygen (1–1.2 μmoles of oxygen per μmole of glucuronic acid formed) and the disappearance of an equivalent amount (± 10%) of inositol (determined by bioassay[3]).

Acknowledgment.—This work was supported by grant No. C-2228(C2) from the National Institutes of Health, Public Health Service, Bethesda, Maryland.

(1) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

(2) F. G. Fischer and H. Dörfel, *ibid.*, **301**, 224 (1955).

(3) L. Atkin, A. S. Schultz, W. L. Williams and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF MEDICINE
UNIVERSITY OF PENNSYLVANIA
PHILADELPHIA, PENNSYLVANIA

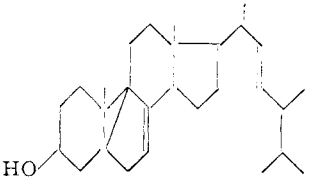
FRIXOS C. CHARALAMPOUS
CHRYSSOULA LYRAS

RECEIVED MARCH 23, 1957

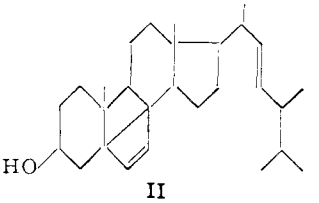
THE STRUCTURE OF PHOTOISOPYROCALCIFEROL[1]

Sir:

Recent studies by Velluz, Havinga and their associates[2] have established that the first step in the ultraviolet irradiation of ergosterol (9α-H, 10β-CH_3, 9,10-*anti*) is a bond cleavage reaction with the formation of a 9,10-*seco*-sterol type of intermediate. On the other hand, Dimroth and Windaus[3] have shown that a similar irradiation of the 9,10-*syn* epimer, isopyrocalciferol (9β-H, 10β-CH_3), is a bond forming reaction. The product, photoisopyrocalciferol, showed the presence of only two double bonds (on quantitative hydrogenation), showed no maximum in the ultraviolet spectrum and upon oxidation yielded a non-conjugated unsaturated ketone. The photo isomer, upon heating, was reconverted to the starting homoannular diene. On the basis of these data, the following two structures (I and II) were postulated and the present work has established the correctness of structure II.



I



II

(1) This work was supported, in part, by Grant A-709 (C4)-Bio (5) of the U. S. Public Health Service, National Institutes of Health, Department of Health, Education and Welfare.

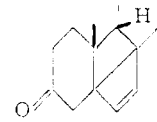
(2) L. Velluz, G. Amiard and B. Goffinet, *Compt. rend.*, **240**, 2326 (1955), and earlier papers; E. Havinga, A. Verloop and A. L. Koevoet, *Rec. trav. chim.*, **75**, 371 (1956).

(3) K. Dimroth, *Ber.*, **70**, 1631 (1937); A. Windaus, K. Dimroth and W. Breywisch, *Ann.*, **543**, 240 (1940).

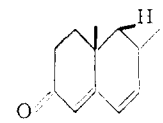
Photoisopyrocalciferol displays bands in the infrared at 970 and 748 cm.$^{-1}$, characteristic of *trans*- and *cis*-symmetrically disubstituted olefins, respectively, and the nuclear magnetic resonance spectrum shows vinyl proton bands at 0 and −1.0 (doublet) p.p.m.[4] The former band is characteristic of the 22-ene structure and thus the latter must be assigned to the remaining nuclear double bond. The large negative displacement is similar to that found for the vinyl proton in cyclobutene (−0.72 p.p.m.).[5] Upon oxidation, the photo compound is converted into a crystalline non-conjugated unsaturated ketone (m.p. 80–81°; C, 85.36; H, 10.62; $\lambda_{max.}$ 1705 cm.$^{-1}$; ϵ_{205} 2350, no. max. at higher wave length) which upon reduction by lithium aluminum hydride is converted to the starting alcohol. Upon ozonization of the acetate, there is obtained a tricarboxylic acid (m.p. 273–275°, C, 64.12; H, 7.51, neut. equiv. 157) which is readily transformed into a cyclic anhydride-carboxylic acid (m.p. 243–244°, C, 66.78; H, 7.62; $\lambda_{max.}$ 1770 and 1825 cm.$^{-1}$). The photo compound can be hydrogenated, stepwise, to yield a dihydro (m.p. 58–59°; C, 84.46; H, 11.58) and a tetrahydro alcohol (m.p. 51.0–52.5°; C, 84.02; H, 12.01); the nuclear double bond is attacked first. All of the foregoing compounds are stable to hydrogen chloride in chloroform solution.[6] These data clearly establish the presence of olefinic linkages at C_6,C_7 and C_{22},C_{23} and they also indicate the presence of a cyclobutene ring and the absence of a cyclopropane structure.

The tetrahydro alcohol upon oxidation with CrO_3 in acetic acid at 0° yields a ketone (m.p. 47.0–48.5°; C, 84.51; H, 11.47; $\lambda_{max.}$ 1705 cm.$^{-1}$; ϵ_{205} 680) whose infrared and ultraviolet spectra are normal for a saturated, isolated ketone. Either the tetrahydro alcohol or ketone upon oxidation with CrO_3 in acetic acid at 70°, followed by esterification with diazomethane, yields a diester (m.p. 65.0–66.5°; C, 77.68; H, 8.47; sapon. equiv., 229), showing the presence of at least one adjacent methylene group. The tetrahydro ketone upon reaction with perbenzoic acid gives rise to a lactone (m.p. 71–73°; C, 81.30; H, 11.31) which when saponified yields a hydroxy acid (m.p. 172–173°; C, 77.59; H, 11.01). Oxidation of the latter compound with CrO_3 in acetic acid at 60° forms the same dibasic acid obtained directly from the ketone, thus indicating the absence of substituents α to the carbonyl group.[7]

When the ketone III derived from the photo compound is allowed to stand with alcoholic alkali, it is converted into oily 4,6-dien-3-one, IV, ($\lambda_{max.}^{EtOH}$ 283 mμ, ϵ 21,00; $\lambda_{max.}$ 1650 cm.$^{-1}$) of the isopyrocalciferol series which can be characterized as a semicarbazone (m.p. 221–223°; C, 77.21; H, 10.12; N, 9.47; $\lambda_{max.}^{EtOH}$ 305 mμ, ϵ 38,500). The same compounds also can be prepared directly from isopyrocalciferol by oxidation followed by base and acid isomerization.



III



IV

Finally, the photo compound II upon heating to 160° in EtOD is reconverted into isopyrocalciferol which is devoid of deuterium. The above facts are only consistent with the valence tautomeric structure II for photoisopyrocalciferol.

Photopyrocalciferol also has been subjected to a similar sequence of reactions and shown to possess an analogous-type structure, different only in the stereochemistry at C_9-C_{10}. This formation of the valence tautomeric structures from 9,10-*syn* structures calls attention to the importance of stereochemistry in the irradiation of 5,7-dienes.

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF CALIFORNIA
BERKELEY 4, CALIFORNIA
WILLIAM G. DAUBEN
GERHARD J. FONKEN

RECEIVED APRIL 24, 1957

(4) Parts per million displacement relative to ethanol.

(5) J. D. Roberts and A. T. Bottini, private communication.

(6) D. H. R. Barton, J. E. Page and E. W. Warnhoff, *J. Chem. Soc.*, 2715 (1954).

(7) Preliminary studies of deuterium exchange with the tetrahydro ketone indicate the presence of 4 enolizable hydrogen atoms; the deuterium analyses were kindly performed by Dr. N. R. Trenner, Merck, Sharp and Dohme Laboratories.)

KINETIC ISOTOPE EFFECT IN THE IODINATION OF 2,4,6-TRIDEUTEROPHENOL

Sir:

The bromination of benzene and bromobenzene, and of their tritiated analogs as catalyzed by iodine proceeds without preferential displacement of hydrogen over tritium[1]; likewise bromination of benzene and hexadeuterobenzene by hypobromous acid with an acid catalyst proceeds at identical rates for these two compounds under similar reaction conditions.[2] Similarly no kinetic isotope effect has been found for nitration[1,3] of benzene, toluene, bromobenzene, naphthalene, or nitrobenzene containing tritium or deuterium nor for azo coupling with 1-naphthol-4-sulfonic acid.[4] The conclusion can be drawn that the breaking of the C–H bond has not made much progress in the transition state of the slow step of these electrophilic substitutions and, with less certainty,[5] that an intermediate[6] ArH·E, is formed during this slow step with electrophilic reagent E with loss of the proton occurring in a subsequent fast step.

While the above examples appear to be the only cases in which it has been proven that loss of the proton is kinetically insignificant during electrophilic aromatic substitution, it has been tempting to apply this generalization to other cases. In particular Painter and Soper[7] and Berliner[8] have implicitly or explicitly made this assumption in

(1) L. Melander, *Arkiv Kemi*, **2**, 211 (1950); *Acta Chem. Scand.*, **3**, 95 (1949).

(2) P. B. D. de la Mare, T. M. Dunn and J. T. Harvey, *J. Chem. Soc.*, 923 (1957).

(3) T. G. Boner, F. Bowyer, and G. Williams, *ibid.*, 2650 (1953); W. M. Lauer and W. E. Noland, THIS JOURNAL, **75**, 3689 (1953).

(4) H. Zollinger, *Helv. Chim. Acta*, **38**, 1597, 1617 (1955).

(5) G. S. Hammond, THIS JOURNAL, **77**, 334 (1955).

(6) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 279 ff.

(7) B. S. Painter and F. G. Soper, *J. Chem. Soc.*, 342 (1947); F. G. Soper and G. F. Smith, *ibid.*, 2757 (1927).

(8) E. Berliner, THIS JOURNAL, **73**, 4307 (1951).