STEROL INTERMEDIATES IN THE CONVERSION OF CHOLESTEROL INTO PREGNENOLONE

Seymour Lieberman, Lajos Bandy, Violet Lippman and Kenneth D. Roberts

Departments of Biochemistry and of Obstetrics and Gynecology
College of Physicians and Surgeons of Columbia University
New York, N. Y.

Received January 6, 1969

The reaction of 20α-hydroxycholesterol-3-acetate with lead tetraacetate has led, probably via free radical intermediates, to the formation of pregnenolone acetate in 67% yield. In view of the fact that the identity of the intermediates in the biological conversion of cholesterol into pregnenolone has not been established with certainty, this fragmentation reaction may be considered to serve as a model for the biological processes which lead to the degradation of the C-27 sterol to the C-21 ketone.

We recently have demonstrated that 20α-hydroxycholesterol and its sulfate, when incubated with sonicated adrenal mitochondria, are converted in good yield into pregnenolone and pregnenolone sulfate, respectively (Roberts et al., 1969). In spite of this, we and others (Koritz and Hall, 1964; Hall and Koritz, 1964; Simpson and Boyd, 1967) have been unable to "trap" radioactivity in this hydroxysteroid when labeled cholesterol is used as substrate for this conversion. This failure to isolate or to obtain information about the nature of the intermediates between cholesterol and pregnenolone has led us to consider the possibility that the intermediates may resemble some of the transitory free radicals involved in the autooxidation of isobutane (Mayo, 1968). The intermediates in this reaction are postulated to be: ($R=CH_3$)

$$R_3CH \longrightarrow R_3C\cdot \longrightarrow R_3C\text{-}O\text{-}O\cdot \longrightarrow$$

$$R_3C\text{-}O\cdot \xrightarrow{\text{fragmentation}} R_2C{=}O + R\cdot$$

In the enzyme-catalyzed reactions involved in the conversion of cholesterol into pregnenolone, the analogous formulation would involve an interaction between the reactants: - the sterol, the specific enzyme, oxygen and cytochrome P 450 (in the presence of an electron transport chain consisting of TPNH, a flavoprotein and a specific non-heme iron protein) to form a product that behaves like alkoxy radicals $\rangle C\text{-}O\cdot$, in that it is able to fragment by cleavage of a C-C bond. In this instance, either spontaneously or through enzymic intervention, the products of fragmentation would be pregnenolone and a six-carbon moiety that is usually isolated as isocaproic acid. This formulation would provide an explanation for the failure to isolate intermediates in the conversion or to "trap" isotope in 20α-hydroxycholesterol. In this scheme, 20α-hydroxycholesterol is not an intermediate even though, by its ready conversion into a product which behaves like an alkoxy radical, it may be efficiently converted into pregnenolone. That oxygen radicals are involved in some biological oxidations and hydroxylations is well accepted (Staudinger et al., 1965; Ullrich and Staudinger, 1966) and it would, therefore, seem reasonable to expect that other biological intermediates, in this case formed by interaction of the sterol with an oxygen radical, would possess chemical properties similar to those of simpler organic free radicals.

In order to obtain support for this postulation, we treated 20α-hydroxycholesterol-3-acetate with $Pb(OAc)_4$, a reagent which has been shown to abstract H· from hydroxyl groups (Amorosa et al., 1962) and obtained, in good yield, pregnenolone acetate which undoubtedly arose by fragmentation of the alkoxy radical.

Experimental: To 20 ml of anhydrous benzene, were added 400 mg freshly-prepared lead tetraacetate, 80 mg of calcium carbonate and 200 mg of 20α-hydroxycholesterol-3-acetate. The mixture was heated under reflux for 18 hours under anhydrous conditions after which an additional 200 ml of benzene was added. The extract was washed with a solution of 5% HCl, saturated sodium bicarbonate solution, and with water to neutrality. After drying over anhydrous sodium sulfate, the solution was evaporated to dryness leaving a residue which was purified by chromatography on the reversed phase system: methanol 4, n-propanol 1, water 1.3, toluene 2, isooctane 2; on 160 gm Celite (0.3 ml stationary phase/gm Celite). One hundred seventeen mg of starting material was eluted in hold-back volume 3. A crystalline product (45 mg, 67% yield calculated on consumed reactant), eluted at the end of the first hold-back volume, was recrystallized from methanol: acetone to yield pregnenolone acetate; identified by m.p., by mixed m.p. with an authentic sample and by its infrared spectrum.

This experiment provides a reasonable model for the biological process involved in the conversion of cholesterol into pregnenolone. It affords a rationalization for the failure to isolate the intermediates of this transformation and it explains the ready conversion of 20α-hydroxycholesterol into pregnenolone by adrenal preparations. The traditional representation (Samuels and Uchikawa, 1967) of the biosynthetic degradation of the C-27 sterol to the C-21 ketone usually depicts the hydroxylated sterols, 20α-hydroxycholesterol, 20α,22ζ-dihydroxycholesterol and 22ζ-hydroxycholesterol (Chaudhuri, et al., 1962) as intermediates. None of these compounds has been shown to satisfy the necessary criterion for intermediacy: formation from the natural precursor by a single, purified enzyme.

The evidence adduced for these intermediates consists of: (1) isolation from natural sources (Roberts et al., 1969), (2) transformation into pregnenolone by biological preparations containing the necessary en-

zyme system (Constantopoulos and Tchen, 1961; Shimizu et al., 1961; Shimizu et al., 1962; Constantopoulos et al., 1962; Koritz and Hall, 1964; Roberts et al., 1969), (3) "trapping" isotope in the intermediate when labeled, known precursor was used as substrate in the biological conversion (Solomon et al., 1956; Ichii et al., 1963; 1967), (4) accumulation of the intermediate when the biological conversion was conducted in the presence of an appropriate unlabeled inhibitor (Ichii et al., 1963) and (5) inhibition of the conversion of radioactive cholesterol into labeled pregnenolone when the conversion was conducted in the presence of the unlabeled intermediate (Hall and Koritz, 1964; but see Boyd and Simpson, 1967). Collectively, it might appear that these items of evidence established the identity of the biological intermediates involved in this transformation. However, some of the quoted evidence is in disagreement and not all can be relied upon with equal confidence. In any case, none, even when taken at face value, constitutes certain proof and, in view of the prevailing uncertainty, we believe that it is fitting to call attention to the radical mechanism examined in this paper.

While our work was in progress, van Lier and Smith (1968) reported some findings on the autooxidation of cholesterol that are relevant. They found that the autooxidation of cholesterol (undoubtedly mediated by radical intermediates) led to the formation of several products among which were cholesterol 20α-hydroperoxide, cholesterol-25-hydroperoxide, pregnenolone and dehydroisoandrosterone. These findings suggested to the authors the possibility that the mechanisms characterizing this process might be analogous to those involved in the biosynthesis of the steroid hormones from cholesterol.

ACKNOWLEDGEMENTS

This investigation was supported by NIH Grant No. AM-00110 from the United States Public Health Service.

REFERENCES

Amorosa, M., Caglioti, L., Cainelli, G., Immer, H., Keller, J., Wehrli, H., Mihailovic, M.L., Schaffner, K., Arigoni, D. and Jeger, O., Helv. Chim. Acta, 45, 2674 (1962).

Constantopoulos, G., and Tchen, T.T., Biochem. Biophys. Res. Comm., 4, 460 (1961).

Constantopoulos, G., Satoh, P.S. and Tchen, T.T., Biochem. Biophys. Res. Comm., 8, 50 (1962).

Chaudhuri, A.C., Harada, Y., Shimizu, K., Gut, M. and Dorfman, R.I., J. Biol. Chem., 237, 703 (1962).

Hall, P.F. and Koritz, S.B., Biochim. Biophys. Acta, 93, 441 (1964).

Ichii, S., Forchielli, E. and Dorfman, R.I., Steroids, 2, 631 (1963).

Ichii, S., Omata, S. and Kobayashi, S., Biochim. Biophys. Acta, 139, 308 (1967).

Koritz, S.B. and Hall, P.F., Biochemistry, 3, 1298 (1964).

Mayo, F.R., Accounts of Chem. Research, 1, No. 7, 193 (1968) and references cited therein.

Roberts, K.D., Bandy, L. and Lieberman, S., Biochemistry. In press (1969).

Samuels, L.T. and Uchikawa, T. In "The Adrenal Cortex". Eisenstein, A.B. ed. Boston, Little, Brown & Co., 1967. p. 61.

Shimizu, K., Hayano, M., Gut, M. and Dorfman, R.I., J. Biol. Chem., 236, 695 (1961).

Shimizu, K., Gut, M. and Dorfman, R.I., J. Biol. Chem., 237, 699 (1962).

Simpson, E.R. and Boyd, G.S., Europ. J. Biochemistry, 2, 275 (1967).

Solomon, S., Levitan, P. and Lieberman, S., Rev. Can. Biol., 15, 282 (1956).

Staudinger, H., Kerekjarto, B., Ullrich, V. and Zubrzycki, Z. In "Oxidases and Related Redox Systems". King, T.E., Mason, H.S. and Morrison, M. eds. New York, John Wiley & Sons, Inc., 1965. p. 815.

Ullrich, V. and Staudinger, H. In "Biological and Chemical Aspects of Oxygenases". Proc. U.S. and Japan Symposium on Oxygenases. Bloch, K.E. and Hayaishi, O. eds. Tokyo, Maruzen Co., Ltd., 1966. p. 235.

van Lier, J.E. and Smith, L.L. Amer. Chem. Soc. Abstracts 156th National Meeting. Atlantic City, N.J., 1968.