in the investigations of nitro phenolic compounds[5] and was observed in the present work for the second stage reduction of dinitrobenzenes which represents the reduction of nitrophenylhydroxyl amines. The *o*-isomer which may form H-bonds between the nitro and the hydroxylamine group was more readily reduced than the corresponding meta- or para-isomers.

After the completion of the present work, Pearson[6] published the results of his investigations of the polarography of the nitrotoluenes and the dinitrobenzenes. Pearson using a potassium hydrogen phthalate–hydrochloric acid buffer over the *p*H range 2.5–3.8 and a potassium hydrogen phthalate–sodium hydroxide buffer over the *p*H range 4.1–7.4 reported half-wave potentials which were from about 0.25 to 0.35 volt more positive than those found in this Laboratory using different systems. Some of Pearson's work has been repeated and verified in this Laboratory. It seems improbable that the differences in reduction potentials could be accounted for on the basis of large differences in concentrations of the nitrobenzene derivatives used in the two investigations. It seems more probable that the reduction of the nitro group is a function not only of the *p*H but also of the nature of the buffer. This effect is being further investigated.

Pearson obtained a second wave at low *p*H values which corresponded approximately to a two electron process which, when added to the height of the first wave involving a four electron process, represented the complete reduction to the corresponding aniline. This second small wave has also been observed in this Laboratory at a *p*H of 2.7 for all the substituted nitrobenzenes but it was very poorly defined and so was not included in the tabulated data.

(5) W. P. Cropper and M. J. Astle, THIS JOURNAL, **65**, 2395 (1943).

(6) J. Pearson, *Trans. Faraday Soc.*, **44**, 683 (1948).

Summary

The reduction of the ortho-, meta- and para-isomers of nitrobenzoic acid, dinitrobenzene, chloronitrobenzenes and nitrotoluene from buffered solutions at the dropping mercury cathode have been studied.

Within each group of isomers, the ortho-compound was more difficultly reduced than the para-compound. It is possible to correlate the electronegativity of the second substituent with the reduction potential of the nitro group and to qualitatively determine the effect of position on the degree of the electronegativity of that group.

o-Nitrobenzoic acid does not behave like the other ortho-compounds in that the reduction potential is much more sensitive to differences in *p*H of the solution.

In every case reduction stops with the formation of the corresponding hydroxylamines, with the exception of the dinitrobenzenes and in these cases the complexity of the curves make difficult the determination of the intermediate steps of reduction.

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Sulfate Esters as Intermediates in the Formation of 7-Dehydrocholesterol and Dicholesteryl Ether[1]

BY ALBERT E. SOBEL, PHYLLIS S. OWADES[2] AND JOSEPH L. OWADES[3]

A certain amount of evidence has accumulated pertaining to the importance of sulfate esters in the chemistry of steroids in the living organism. At least six different steroid sulfates have been isolated from urine,[4–9] as well as a dehydrosteroid which was postulated as having been formed from a sulfate ester during the course of isolation.[10] A sulfate containing lipid was found in brain.[11] Recently it has been shown that the human kidneys maintain a definite level of sulfate in the blood. The existence of this threshold suggests that the sulfate in the blood, heretofore considered to be simply excretory, plays some metabolic role.[12,13]

Naturally occurring 7-dehydrocholesterol and dicholesteryl ether are found associated with the relatively abundant cholesterol, from which they would seem to be derived. 7β-Hydroxycholesterol, the probable intermediate in the formation of 7-dehydrocholesterol, has also been isolated.

(1) Presented before the Division of Biological Chemistry of the American Chemical Society at Chicago, Ill., April, 1948.

(2) Abstracted from the thesis submitted to the Faculty of the Graduate School of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Master of Science in Chemistry.

(3) Abstracted from the dissertation which is being submitted to the Faculty of the Graduate School of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(4) Schachter and Marrian, *Proc. Soc. Exp. Biol. Med.*, **35**, 222 (1936).

(5) Munson, Gallagher and Koch, *J. Biol. Chem.*, **152**, 67 (1944).

(6) Venning, Hoffman and Browne, *ibid.*, **146**, 369 (1942).

(7) Klyne and Marrian, *Biochem. J.*, **39**, XLV (1945).

(8) Klyne, *ibid.*, **40**, 875 (1946).

(9) Paterson and Klyne, *ibid.*, **41**, proc. 2 (1947).

(10) Burrows, Cook, Roeb and Warren, *Biochem. J.*, **31**, 950 (1937).

(11) Levene, *J. Biol. Chem.*, **53**, 614 (1912).

(12) Vars and Gurd, *Am. J. Physiol.*, **151**, 399 (1947).

(13) Letspeich, *ibid.*, **151**, 311 (1947).

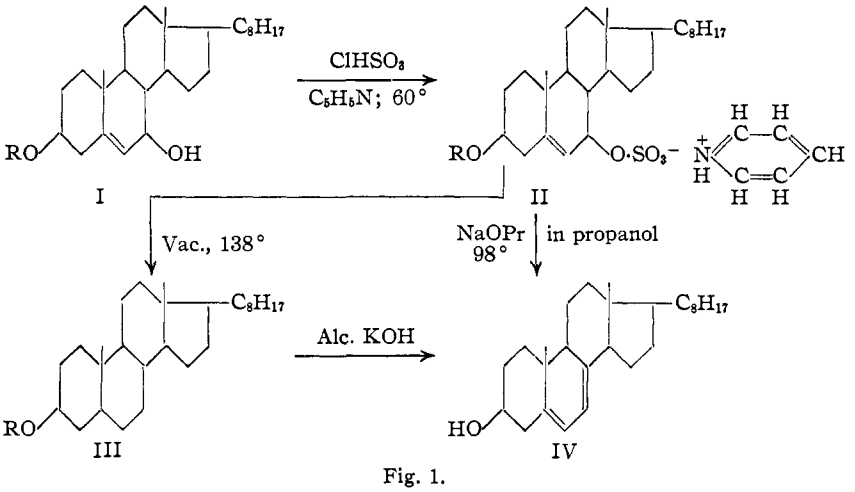

Fig. 1.

In view of this evidence, it seemed possible that sulfate esters might be the metabolic intermediates in the conversion of cholesterol to 7-dehydrocholesterol and dicholesteryl ether. We have shown in this investigation that the formation of these two steroids from their corresponding sulfates is possible *in vitro*.

It had previously been demonstrated that monovalent metal salts of cholesteryl sulfate decompose on heating to give 3,5-cholestadiene.[14,15] It was thus of added interest to determine whether the 7-position in the sterol nucleus underwent a similar reaction.

The method employed in the formation of 7-dehydrocholesterol from cholesterol is shown in Fig. 1. 7β-Hydroxycholesteryl phthalate (I) was made by permanganate oxidation of cholesteryl phthalate using a modification of the method of Heilbron, *et al.*[16] The 7-sulfate (II) was then prepared by treating the alcohol with a mixture of chlorosulfonic acid and pyridine. Decomposition of the sulfate, either in the dry state or in a solvent, resulted in 7-dehydrocholesterol (IV).

This investigation was facilitated by the use of two color reactions which distinguished between 7β-hydroxycholesterol and 7-dehydrocholesterol. The known steroid color tests for 7-dehydro and 7-hydroxy steroids, namely, the Rosenheim,[17] Callow,[18] Lifschutz,[19] and antimony trichloride reactions, give positive tests with both compounds. It was found that the Callow reaction could be modified so as to differentiate between these two steroids. The 7-dehydro compound gave a positive test with this reagent, whereas the 7-hydroxy compound remained colorless. The course of decompositions, as well as purification procedures, were readily followed with this test.

(14) Natelson and Gottfried, THIS JOURNAL, **61**, 971 (1939).
(15) Sobel and Rosen, *ibid.*, **63**, 3536 (1941).
(16) Barr, Heilbron, Parry and Spring, *J. Chem. Soc.*, 1437 (1936).
(17) Rosenheim, *Biochem. J.*, **23**, 47 (1929).
(18) Rosenheim and Callow, *ibid.*, **25**, 74 (1931).
(19) Bergstrom and Wintersteiner, *J. Biol. Chem.*, **145**, 309 (1942).

The activated glycerol dichlorohydrin reagent, which is used in the determination of Vitamin A,[20] was also helpful in distinguishing between these steroids. With this reagent the 7-dehydrocholesterol gave a negative reaction, but 7-hydroxycholesterol gave a dark blue color. This is the only substance which is known to give a blue color with activated glycerol dichlorohydrin.

Dicholesteryl ether was also prepared from sulfate esters. The pyridine radical in pyridinium cholesteryl sulfate was replaced by divalent or trivalent cations, namely, ferrous, ferric and aluminum. These cholesteryl sulfate salts, as well as the calcium salt,[21] decompose at varying temperatures to yield dicholesteryl ether (Fig. 2). The decomposition of the aluminum cholesteryl sulfate occurs spontaneously at room temperature. This low temperature reaction lends support to our hypothesis that sulfate esters are significant in the *in vivo* chemistry of the steroids.

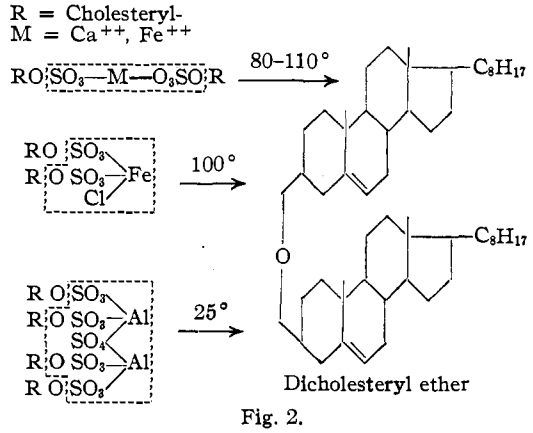

Fig. 2.

It is hoped that future investigations will isolate cholesteryl sulfates and an enzyme system that accomplishes these transformations *in vivo*.

Experimental

7β-Hydroxycholesteryl Phthalate.—A modification of the method of Heilbron, *et al.*,[16] was employed. During a two to three hour period, 1 liter of 0.25 *N* potassium permanganate was added dropwise to a suspension of 10 g. of cholesteryl phthalate in 200 ml. of 1 *N* sodium carbonate. The mixture was vigorously stirred during the addition and for twenty-four hours afterward. Sulfur dioxide was then bubbled in slowly with strong agitation. When completely clarified, the mixture was cooled, filtered, washed with water and dried. The dry solid was extracted with cold

(20) Sobel and Werbin, *Ind. Eng. Chem., Anal. Ed.*, **18**, 570 (1946).
(21) Sobel and Spoerri, THIS JOURNAL, **64**, 482 (1942).

chloroform and ether (1:1). The residue was recrystallized twice from hot pyridine to which water was added until the solution turned cloudy and crystallization began. The yield was 1 g., m. p. 199–201°.

Saponification and recrystallization from methanol gave 7β-hydroxycholesterol, m. p. 178°, $\alpha^{20}D$ −87.3 (in chloroform).

Pyridinium 7-Sulfate Cholesteryl Phthalate.—Chlorsulfonic acid (1.2 g.) was added to a cooled solution of 8 ml. of dry pyridine and 27 ml. of dry chloroform. A suspension of 5.5 g. of 7β-hydroxycholesteryl phthalate in 80 ml. of dry chloroform was introduced. The cold mixture was stirred for twenty minutes, 30 ml. of dry pyridine was added and the solution heated in a water-bath at 60° for two hours. It was vacuum distilled under nitrogen to dryness and washed with cold water until chloride-free. It was then dried in vacuum and recrystallized by dissolving in cold chloroform and adding 8–10 volumes of cold petroleum ether. The yield was quantitative, m. p. 142–144° dec. The Rosenheim, Callow, Lifschutz and antimony trichloride color reactions, which are positive for 7β-hydroxycholesteryl phthalate, are negative for pyridinium 7-sulfate cholesteryl phthalate.

Anal. Calcd. for $C_{40}H_{55}O_8NS$: C, 67.66; H, 7.81; N, 1.97; S, 4.52. Found: C, 68.0; H, 7.62; N, 1.89; S, 4.46.

Decomposition of Pyridinium 7-Sulfate Cholesteryl Phthalate.—A. One-half gram of dry pyridinium 7-sulfate cholesteryl phthalate was placed in an Abderhalden pistol, the pistol evacuated, and xylene (b. p. 138°) refluxed over it for fifteen minutes. Decomposition took place immediately. The product was saponified with 10 ml. of 10% methanolic potassium hydroxide and taken up in peroxide-free ether and water. The ether extract was washed with water, dried over sodium sulfate, and filtered. The ether was blown off with nitrogen and the residue dried in a vacuum desiccator.

B. One-tenth gram of sodium was dissolved in 20 ml. of *n*-propyl alcohol (or ethyl alcohol) contained in a small round bottomed flask with an entering side arm. Pyridinium 7-sulfate cholesteryl phthalate (0.5 g.) was introduced and the solution refluxed for forty minutes. Nitrogen was bubbled through the side arm during the reaction. A brown oil and a white solid separated. The solution was cooled and extracted with ether and water. The water layer gave positive tests for sulfate, phthalate and pyridine. The ether layer, in which the brown oil had dissolved, was dried over sodium sulfate, filtered, and vacuum distilled under nitrogen to dryness.

The residues were shown to be 7-dehydrocholesterol by the absorption spectra, digitonide formation, antirachitic activity, color reactions and the 3,5-dinitrobenzoate derivative.

The yields, as calculated from the molecular extinction coefficient and the weight of the digitonides, were 69% for method A and 30% for method B.

The 3,5-dinitrobenzoate derivative,[22] m. p. 207°, showed no depression with a known sample of the 3,5-dinitrobenzoate of 7-dehydrocholesterol.

Anal. Calcd. for $C_{34}H_{46}O_6N_2$: C, 70.55; H, 8.01; N, 4.84. Found: C, 70.41; H, 7.97; N, 4.96.

Modification of Callow Test.—The reagent[18] (1 ml.) was added to a solution of the steroid (*ca.* 1 mg.) in 4 ml. of alcohol, instead of chloroform as originally proposed. The use of different solvents was first discussed by Sobel.[23] A yellow color was obtained with 7-dehydrocholesterol and no color with 7β-hydroxycholesterol.

Activated Glycerol Dichlorohydrin Test.—Four ml. of reagent[20] was added to a solution of the steroid (*ca.* 1 mg.) in 1 ml. of chloroform. 7β-Hydroxycholesterol gave a deep blue color, the dehydro compound gave no color.

Ferrous Cholesteryl Sulfate.—A solution of 10 g. of pyridinium cholesteryl sulfate[24] in 200 ml. of water was treated with a filtered solution of 10 g. of ferrous sulfate heptahydrate in 100 ml. of water. A white, gelatinous precipitate formed immediately. The suspension was stirred for one-half hour, then filtered and washed with water until the filtrate was free of sulfate and ferrous ions. It was then washed with acetone and dried in a vacuum desiccator. It was insoluble in water, ether or acetone; soluble in pyridine and *n*-amyl alcohol; m. p. 122–125° (dec.).

Anal. Calcd. for $C_{54}H_{90}O_8S_2Fe$: S, 6.50; Fe, 5.66. Found: S, 6.43; Fe, 5.60.

Ferric Cholesteryl Sulfate.—A solution of 13 g. of ferric chloride hexahydrate in 100 ml. of water was filtered and added to 100 ml. of a 5% pyridinium cholesteryl sulfate solution. The mixture was agitated for one-half hour and then centrifuged. The residue was washed with water until free of chloride ion, followed by an acetone and several benzene washes. It was dried in vacuum. The residue was tan colored; insoluble in water, benzene or chloroform; soluble in alcohol; m. p. 115–118° (dec.).

Anal. Calcd. for $C_{54}H_{99}O_8S_2ClFe$: S, 6.27; Fe, 5.46. Found: S, 6.34; Fe, 5.55.

Aluminum Cholesteryl Sulfate.—A solution of 12 g. of pyridinium cholesteryl sulfate in 120 ml. of water was treated with a filtered solution of 30 g. of aluminum sulfate octadecahydrate in 120 ml. of water and stirred for ten minutes. The suspension was filtered and washed with water until the washings were sulfate-free, and then washed with alcohol and dried in a vacuum desiccator. The residue was soluble in pyridine and *n*-butyl alcohol; insoluble in water, alcohol and dioxane; m. p. 115–120° (dec.).

Anal. Calcd. for $C_{108}H_{180}O_{20}S_5Al_2$: S, 7.97; Al, 2.68. Found: S, 7.84; Al, 2.52.

Dicholesteryl Ether.—Pyrolysis of the ferrous, ferric and aluminum cholesteryl sulfates in evacuated glass tubes resulted in the formation of dicholesteryl ether. The temperatures required for rapid decomposition were 138° for the ferric salt, and 115° for the ferrous and aluminum salts. The aluminum salt, in addition, was found to undergo this change in several weeks at room temperature. The decomposed material was extracted with warm benzene and filtered from the inorganic residue. The benzene was evaporated and the resinous mass that remained was leached with a mixture of ether and alcohol (1:1). The residue was dissolved in benzene and precipitated with alcohol or acetone. This was repeated several times and the final residue was washed with alcohol and dried; m. p. 205–207°. It gave no depression with a known sample of dicholesteryl ether. The tetrabromide derivative[25] melted at 166°.

Summary

A sulfate ester of a 7-hydroxy steroid, the pyridinium sulfate of 7β-hydroxycholesteryl phthalate, was prepared. It decomposed to 7-dehydrocholesterol, analogously to a known reaction of 3-sulfate esters.

Two color reactions to distinguish between 7-hydroxy and 7-dehydro steroids were given, which, to the authors' knowledge, are the first reactions to accomplish this differentiation.

Dicholesteryl ether was obtained from ferrous, ferric and aluminum cholesteryl sulfates; at room temperature from the latter compound.

Based on the above findings and a survey of the literature, it is suggested that sulfate esters might be the metabolic intermediates in the conversion of cholesterol to 7-dehydrocholesterol and dicholesteryl ether.

RECEIVED NOVEMBER 19, 1948

(22) Windaus, Lettre and Schenck, *Ann.*, **520**, 98 (1935).

(23) Sobel, Dissertation, Ph.D., Polytechnic Institute of Brooklyn, 1940.

(24) Sobel and Spoerri, THIS JOURNAL, **63**, 1259 (1941).

(25) Levin, *ibid.*, **65**, 627 (1943).