

**Metal Ion Catalyzed Oxidation of Steroids. II.¹⁾ Oxidation of Cholesterol
by Fenton's Reagent in Acetic Acid Solution²⁾**

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When cholesterol (I) was treated with the hydrogen peroxide-ferrous sulphate system in an acetic acid solution, there were formed 5 α -cholestane-3 β ,5,6 β -triol (II), 3 β -acetoxy-5 α -cholestane-5,6 β -diol (III), 5,6 α -epoxy-5 α -cholestan-3 β -ol (IV), 6 β -acetoxy-5 α -cholestane-3 β ,5-diol (V), and 3 β ,6 β -diacetoxy-5 α -cholestan-5 α -ol (VI). Ferrous ions accelerated remarkably the formation of these products (Fig. 2) as well as peracetic acid (Fig. 3). The same ions were, however, likely to be independent of forming the epoxide (IV) from I with peracetic acid (Fig. 4). Hydrogen peroxide and ferrous ions were cooperative in facilitating the formation of II from IV, contrary to be entirely invalid in their independent use (Fig. 5). Thus, it seemed in the present reaction that the epoxide (IV) is produced heterolytically, as usual, with peracetic acid which can easily be formed in the mixture of acetic acid and hydrogen peroxide (Chart 1) and that the triol (II) as well as the derivatives (III, V, and VI) are formed mainly through IV.

It has long been postulated that hydroxyl radicals are produced when a ferrous salt is added to an aqueous solution of hydrogen peroxide.⁴⁾ Since Fenton's reagent,⁵⁾ hydrogen peroxide-ferrous sulphate system, was found to be capable of hydroxylating benzene to give phenol and polyphenols with great ease at room temperature,⁶⁾ the action of the reagent on a number of organic compounds has been extensively studied by many investigators. In 1950, Clemo, *et al.*⁷⁾ reported the formation of 5 α -cholestane-3 β ,5,6 β -triol (II)⁸⁾ and 3 β -acetoxy-5 α -cholestane-5,6 β -diol (III) from cholesterol (I) by Fenton's reagent in a dilute aqueous acetic acid medium. They understood these results as a general tendency for the hydroxyl radicals to be added to the isolated double bond in the C-5:6 position. In a series of studies on the metal-catalyzed oxidation of steroidal compounds, the present authors reinvestigated the Clemo's experiment and found the formation of 5,6 α -epoxy-5 α -cholestan-3 β -ol (IV)⁹⁾ besides the triol (II), III, 6 β -acetoxy-5 α -cholestane-3 β ,5-diol (V),¹⁰⁾ and 3 β ,6 β -diacetoxy-5 α -cholestan-5-ol (VI).⁸⁾ This paper deals with the hydroxylation of cholesterol by Fenton's reagent in an aqueous acetic acid solution and with the roles played by ferrous ions during course of the reaction.

- 1) Part I: M. Kimura, M. Kawata, M. Tohma, A. Fujino, K. Yamazaki, and T. Sawaya, *Chem. Pharm. Bull.* (Tokyo), **20**, 1883 (1972).
- 2) This work was presented at the 91st Annual Meeting of the Pharmaceutical Society of Japan, April 1971, Fukuoka.
- 3) Location: *Nishi-6-chome, Kita-12-jo, Sapporo.*
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- 7) C.R. Clemo, M. Keller, and J. Weiss, *J. Chem. Soc.*, **1950**, 3470.
- 8) L.F. Fieser and S. Rajagopalan, *J. Am. Chem. Soc.*, **71**, 3938 (1949).
- 9) L.F. Fieser and M. Fieser, "Reagents for Organic Synthesis," John Wiley and Sons, Inc., New York, 1967, p. 136.
- 10) B. Ellis and V. Petrow, *J. Chem. Soc.*, **1939**, 1078.

Result and Discussion

Products Analysis

When hydrogen peroxide and ferrous sulphate were added dropwise to a vigorously stirred acetic acid solution of cholesterol (I), there were formed 5 α -cholestane-3 β ,5,6 β -triol (II), 3 β -acetoxy-5 α -cholestane-5,6 β -diol (III), 6 β -acetoxy-5 α -cholestane-3 β ,5-diol (V), and 3 β ,6 β -diacetoxy-5 α -cholestan-5-ol (VI). These products were isolated from the reaction mixture by the column chromatography on alumina and were identified individually with the authentic specimens (Table I). In addition to them, the formation of 5,6 α -epoxy-5 α -cholestan-3 β -ol (IV) which Clemo, *et al.*⁷⁾ failed to obtain was also recognized by thin-layer as well as gas-liquid chromatography of the reaction mixture at the earlier period (Fig. 1). Although the identification was thus done merely in comparative ways with the authentic specimen of IV and the isolation was unsuccessful because of its lower stability in acidic medium, these results seemed to be interpreted to indicate that the hydroxylation of I proceeds through the intermediary formation of the epoxide (IV).

TABLE I. Column Chromatography of Reaction Products

Fraction No.	Solvent	Vol. (liter)	Weight (mg)	R _f on TLC
1	hexane-benzene (1:1)	1.5	224	0.55 (VI)
2	benzene	0.7	162	0.45 (III), 0.55 (VI)
3	benzene-chloroform (95:5)	1.0	242	0.20 (V), 0.45 (III)
4	benzene-chloroform (70:30)	0.4	347	0.20 (V)
5	benzene-chloroform (40:60)	1.5	129	0.02 (II), 0.20 (V)
6	chloroform-methanol (90:10)	0.5	163	0.02 (II)
	chloroform-methanol (50:50)	1.0		

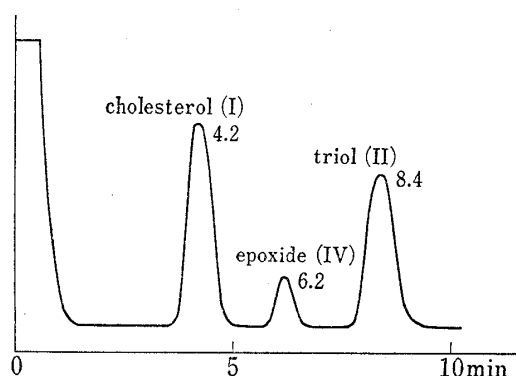


Fig. 1. Gas Chromatogram of Reaction Mixture^{a)} at the Earlier Period

column: 1.5% SE-30 on Shimalite W; column temperature: 240°; carrier gas: N₂ at 60 ml/min

a) Acetates (III, V, and VI) were hydrolyzed to the triol (II) in advance, as described in the experimental part.

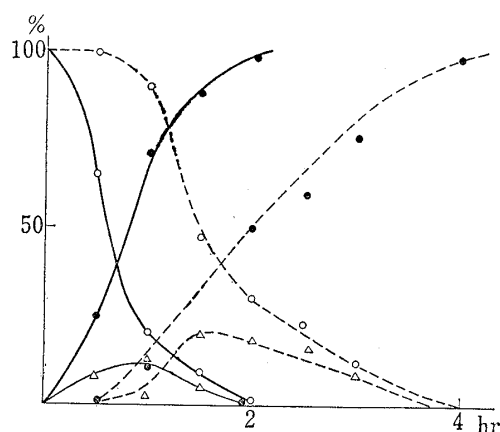


Fig. 2. Effect of Ferrous Ions on the Hydroxylation

—○— : cholesterol (I)
 —●— : triol (II)^{a)}
 —△— : epoxide (IV)
 — : AcOH-H₂O₂-Fe²⁺
 - - - : AcOH-H₂O₂

a) Acetates (III, V, VI) were hydrolyzed to the triol (II) in advance, as described in the experimental part.

Effects of Ferrous Ions on the Hydroxylation

A remarkable difference in the rate of hydroxylation was observed between the reaction mixture with ferrous ions and that without the ions, as shown in Fig. 2. Although the hydroxylation proceeded under absence of ferrous ions, the consumption of I in the reaction mixture was doubly accelerated by the presence of ferrous ions. Fenton's reagent has generally been postulated as the hydroxyl radical generator⁴⁾ and Clemo, *et al.*⁷⁾ assumed the direct attack of the radical to the double bond in I to form II and III. Keller and Weise¹¹⁾ isolated II and 3β -hydroxycholest-5-en-7-one (VII) from I in the irradiated aqueous solutions, though they failed to obtain the epoxide. By using Fenton's reagent, Hamilton, *et al.*¹²⁾ was capable of obtaining the epoxide from cyclohexene-substrate.

Epoxides, on the other hand, can also be formed heterolytically by the peracid¹³⁾ and the possibility cannot be exclusive that IV is produced from I with peracetic acid which is one of the most useful epoxidizing agents and can be readily formed in a mixture of acetic acid and hydrogen peroxide. Formation of the peracid both in the presence and in the absence of ferrous ions was thus determined iodimetrically¹⁴⁾ under the presence of ammonium bifluoride which is an effective masking agent for ferric ions in the titration.¹⁵⁾ As shown in Fig. 3, ferrous ions highly accelerated the formation of peracetic acid. In order to observe the role played by ferrous ions on the epoxidation with peracetic acid, two kinds of cholesterol solution, containing and lacking the ions, were then treated with the peracid prepared in advance. Ferrous ions seemed to be independent of forming the epoxide (IV) in this experiment as shown in Fig. 4. When the reaction of I with the ferrous ions-hydrogen peroxide system was carried out in an acetonitrile medium which can hardly be assumed to form the peracid under the conditions attempted, traces of II, IV, 7α -, and 7β -hydroxy- 5α -cholest-

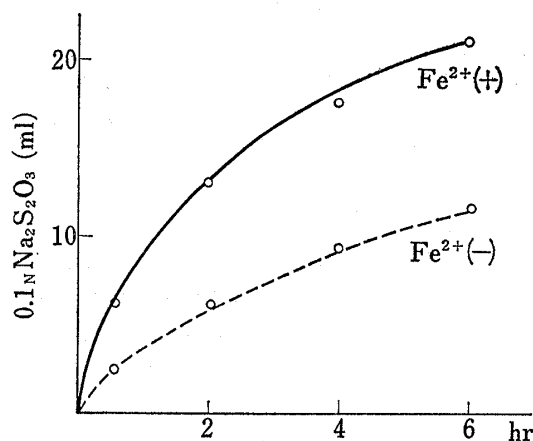


Fig. 3. Effect of Ferrous Ions on Formation of Peracetic Acid

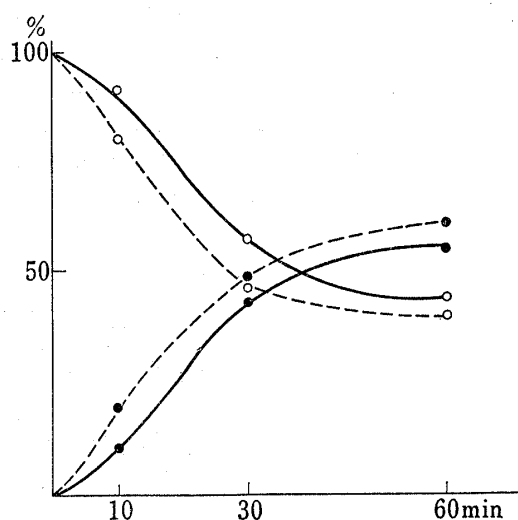


Fig. 4. Epoxidation with Peracetic Acid

○ : cholesterol (I)
● : epoxide (IV)
— : with Fe^{2+}
- - - : without Fe^{2+}

- 11) M. Keller and J. Weise, *J. Chem. Soc.*, **1950**, 2709.
- 12) G.A. Hamilton, R.J. Workman, and L. Woo, *J. Am. Chem. Soc.*, **86**, 3390 (1964).
- 13) a) E.S. Gould, "Mechanism and Structure in Organic Chemistry," H. Holt and Co., New York, 1959, p. 534; b) W.A. Waters, "Mechanism of Oxidation of Organic Compounds," Methuen and Co., Ltd., London 1964.
- 14) W.C. Smit, *Rec. Trav.*, **49**, 675 (1930).
- 15) Japan Society for the Promotion of Science, "Rapid Method of Analysis of Copper in Steel," 1956, p. 104.

5-en-3 β -ol were obtainable,¹⁶⁾ in contrast with the case employing acetic acid medium. From these results, it may be concluded that ferrous ions act on the stage 1 of the formation of peracetic acid (Chart 1) and that the heterolytic epoxidation is predominant in the Clemo's conditions.⁷⁾

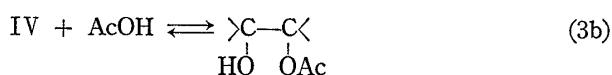
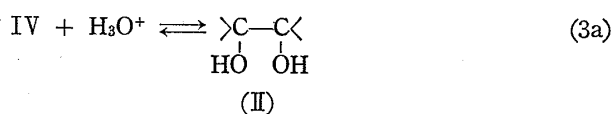
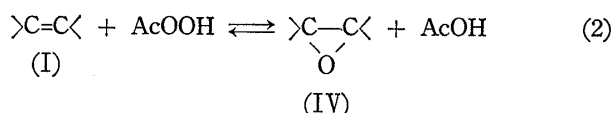


Chart 1

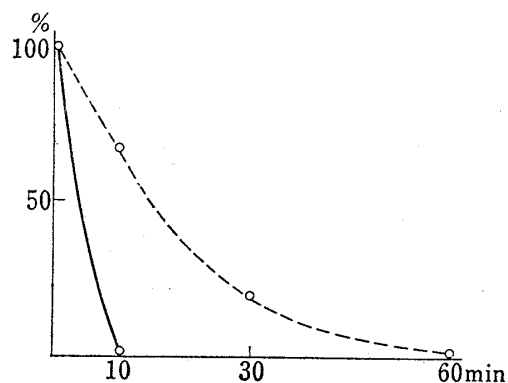


Fig. 5. Transformation of Epoxide to Triol

— : in the presence of Fe^{2+} and H_2O_2
 - - - : when lacking Fe^{2+} , H_2O_2 , or both of them

Formation of the Triol (II) from the Epoxide (IV)

Epoxides are generally assumed to be decomposed by the transition metal ions.¹⁷⁾ Neither ferrous ions nor hydrogen peroxide, however, affected the transformation of the epoxide (IV) to the triol (II) in an acetic acid solution where they were used individually or lacking both of them. Instead, it was surprisingly accelerated by their mixture, the Fenton's reagent,⁵⁾ as shown in Fig. 5. It seemed that ferrous ions are highly effective also for the stage 3 (Chart 1) of the formation of II from IV when hydrogen peroxide is cooperative in the system. Recently, epoxidation reaction with hydrogen peroxide catalyzed by the metal chelate compound was studied on some olefins in benzene-*n*-pentanol solutions and it was found that the opening of the oxirane ring to the α -hydroxy-hydroperoxide is exothermic and very fast.¹⁸⁾ No evidence, however, has been obtainable in the present study for the formation of the corresponding hydroperoxide. The homolytic mechanism might be assumed in the opening of the epoxide ring of IV as well as in the formation of acetylated products (III, V, and VI), as discussed in the study of the irradiated aqueous acetic acid solutions of I.¹¹⁾ In an acetonitrile solution of I, the Fenton's reagent gave 7 α - and 7 β -hydroxycholest-5-en-3 β -ol, though traces in amount.¹⁶⁾ Since hydroxyl radicals can attack the allylic position,^{4f,13b)} the non-stereospecific formation of these diols might also be ascribed to such species. Further discussion, however, is not appropriate until more evidence has been accumulated. Thus, it may be concluded from the data presented here that, when I was oxidized with Fenton's reagent in an acetic acid solution, (1) the formation of the triol (II) and its derivatives through the epoxide (IV) is the main route of the reaction, (2) the epoxide (IV) is formed with peracetic acid mainly in an usual heterolytic mechanism, (3) ferrous ions accelerate the formation of peracetic acid, and (4) ferrous ions and hydrogen peroxide are cooperative in accelerating the formation of II from IV, contrary to be invalid in their independent use.

16) M. Kimura, M. Tohma, and T. Tomita, in preparation.

17) a) W. Sager, *J. Am. Chem. Soc.*, **78**, 4970 (1956); b) R. Stewart, "Oxidation Mechanism," W.A. Benjamin, New York, 1964.

18) A.M. Mattucci, E. Perrotti, and A. Santambrogio, *Chem. Commun.*, **1970**, 1198.

Experimental¹⁹⁾

Materials and Authentic Specimens—Cholesterol (I) and 5 α -cholestane were of commercial sources and purified by recrystallization. The authentic specimens of 5 α -cholestane-3 β ,5,6 β -triol (II),⁸⁾ 5,6 β -epoxy-5 α -cholestan-3 β -ol (IV),⁹⁾ and 3 β ,6 β -diacetoxy-5 α -cholestan-5-ol (VI)^{7,8)} were prepared and purified as reported.

Reactions with Fenton's Reagent—i) In a Preparative Scale: To a stirred solution of cholesterol (I) (2 g, 5.17×10^{-3} mole) in glacial acetic acid (500 ml), was added dropwise a mixture of 30% H₂O₂ (50 ml, *ca.* 4.9×10^{-1} mole), FeSO₄·7H₂O (9.2 g, 3.3×10^{-2} mole), and water (100 ml) during 1 hr at 40° and the reaction mixture was kept at room temperature overnight. Evaporation of the solvent from the mixture at 35–40° *in vacuo* left a residue, which was extracted with ether (400 ml) after adding dil. HCl (50 ml). After acid layer was extracted with fresh ether (400 ml), the combined organic layer was washed with 5% BaHCO₃, then with water and dried over anhydrous Na₂SO₄. Evaporation of ether left the pale yellow powder (1.5 g).

ii) For the Measurement of the Rate of Hydroxylation: To a stirred mixture of I (200 mg, 5.17×10^{-4} mole), glacial acetic acid (50 ml), and an aqueous solution (10 ml) of FeSO₄·7H₂O (920 mg, 3.31×10^{-3} mole), was added 30% H₂O₂ (5 ml, *ca.* 4.9×10^{-2} mole) dropwise during 10 min at 40°. Sampling of the test solutions (5 ml each) was made at the elapsed times of 0.5, 1.0, 2.0, 3.0, and 4.0 hr from the first drop of H₂O₂. Aqueous solution (10 ml) of Na₂SO₃ (1 g) was added promptly to each sample solution and the mixture was extracted twice with fresh ether (10 ml). The organic layer was washed with 10% Na₂CO₃ and with water and dried over anhydrous Na₂SO₄. Evaporation of the solvent from the ether solution left a residue which was submitted to GLC for the estimation of each component. The comparative reaction mixture lacking FeSO₄ was treated also in the same procedure as described above. The rates of hydroxylation as a function of reaction time are shown in Fig. 2.

Purification and Identification of the Products

Column Chromatography—The pale yellow powder (1.5 g) obtained above was submitted to column chromatography on neutral alumina (100–150 mesh, 45 g) using the solvent systems of hexane–benzene, benzene, benzene–CHCl₃, and CHCl₃–MeOH. The results are summarized in Table I.

3 β ,6 β -Diacetoxy-5 α -cholestan-5-ol (VI)—Evaporation of the solvent *in vacuo* from the fraction 1 left a residue (224 mg), which was crystallized from MeOH to give colorless needles, mp 169–170°. IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3470 (OH), 1732, 1713 (C=O), 1263, 1240 (C–O–C). NMR (CDCl₃): 9.33 (18-CH₃), 8.88 (19-CH₃), 8.01 (3-OAc), 7.96 (6-OAc), 4.85 (3-H), 5.30 (6-H). No depression of melting point was observed on admixture with the authentic specimen.^{7,8)}

3 β -Acetoxy-5 α -cholestane-5,6 β -diol (III)—The contents of the fraction 2 were submitted again to preparative TLC and the crude substance thus separated was recrystallized from CHCl₃–MeOH to give colorless flakes, mp 208–210°. IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3460 (OH), 1716 (C=O), 1265 (C–O–C).

6 β -Acetoxy-5 α -cholestane-3 β ,5-diols (V)—Evaporation of the solvent *in vacuo* from the fraction 4 left a residue (347 mg), which was crystallized from MeOH to give colorless needles, mp 144.5–145.5°. IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3480, 3358 (OH), 1723 (C=O), 1272 (C–O–C).

5 α -Cholestane-3 β ,5,6 β -triol (II)—Evaporation of the solvent *in vacuo* from the fraction 6 left a residue (163 mg), which was recrystallized from MeOH to give colorless needles, mp 236–242°. IR $\nu_{\max}^{\text{Nujol}}$ cm⁻¹: 3358 (OH). NMR (pyridine): 9.27 (18-CH₃), 8.78 (19-CH₃). No depression of melting point was observed on admixture with the authentic specimen.⁸⁾

Gas-Liquid Chromatography—The apparatus used was a Shimadzu Model GC-4APF gas chromatograph equipped with a hydrogen flame ionization detector and a U-shaped stainless steel tube (3 m \times 3 mm *i.d.*) packed with 1.5% SE-30 on Shimalite W (60–80 mesh). The column and injection as well as detection temperatures were kept at 240° and 260°, respectively. Nitrogen was used as a carrier gas at a flow rate of 60 ml per min. The samples (3–10 mg) were first refluxed with 3% methanolic KOH (10 ml), in order to hydrolyze the acetyl derivatives involved. After evaporation of MeOH *in vacuo* at 40°, water (5 ml) was added to the residue and extracted with fresh ether (10 ml \times 2). The organic layer was washed with water, dried over anhydrous Na₂SO₄ and concentrated to dryness *in vacuo*. About 1 mg of the residue thus obtained was dissolved in pyridine (0.2 ml) and heated with hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml) at 50° for 15 min.²⁰⁾ After the solvents were blown off to dryness with N₂ stream, the residue was extracted with acetone (0.2 ml) and centrifuged. About 1 μ l of the supernatant was submitted to GLC. Determination of the individual component was made by the half-width method and cor-

19) Melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectral measurements were run on JASCO Model IR-S spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained by Hitachi Model H-6013 spectrometer at 60 Mc. Thin-layer chromatography (TLC) was carried on silica gel (WAKOGEL B-5) plate by the solvent system of AcOEt–cyclohexane (1:1) and by staining with 2N H₂SO₄ and heating at 130° for 5 min.

20) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).

rected by using the following relative sensitivities obtained in advance: 5 α -cholestane (1.00), cholesterol (I) (0.96), 5,6 α -epoxy-5 α -cholestan-3 β -ol (IV) (0.54), 5 α -cholestan-3 β ,5,6 β -triol (II) (0.84). The figures thus obtained were expressed in percentage to the total amount of the components presented in the chromatogram.

Determination of Peracetic Acid—To a mixture of glacial acetic acid (30 ml), water (6 ml), and FeSO₄·7 H₂O (552 mg, 1.99 × 10⁻³ mole), was added 30% H₂O₂ (3 ml, *ca.* 2.9 × 10⁻² mole) dropwise during 10 min. Sampling of the test solutions (5 ml each) was made at the elapsed times of 0.5, 2.0, 4.0, and 6.0 hr after adding H₂O₂. Each test solution was mixed with 4N H₂SO₄ (50 ml) and H₂O₂ remained was titrated with 1/10N KMnO₄ at 0°. NH₄F·HF (4.5 g) and saturated aqueous solution (2.5 ml) of KI were added to the test solution thus obtained, which was allowed to stand for 30 min at a dark place and then titrated with 1/10N Na₂S₂O₃. The comparative reaction mixture lacking FeSO₄ was treated also in the same way as described above. The formation-rates of peracetic acid as a function of reaction time are shown in Fig. 3.

Epoxidation with Peracetic Acid—The mixture of acetic acid (250 ml), water (50 ml), and 30% H₂O₂ (25 ml) was allowed to stand for 24 hr at room temperature. To the stirred equilibrium mixture (30 ml) of peracetic acid thus produced, was added I (200 mg, 5.17 × 10⁻⁴ mole) and FeSO₄·7H₂O (920 mg, 3.31 × 10⁻³ mole). Sampling of the test solutions (5 ml each) was made at the elapsed reaction times of 10, 30, and 60 min. After adding 10% Na₂SO₃ (5 ml) promptly to each sample solution, the mixture was extracted twice with fresh ether (10 ml). The organic layer was washed with 10% Na₂CO₃ and with water and dried over anhydrous Na₂SO₄. Evaporation of the solvent from the ether solution left a residue which was then submitted to GLC for the estimation of epoxide formed. The comparative reaction mixture lacking FeSO₄ was treated also in the same way as described above. The formation-rates of epoxide as a function of reaction time are shown in Fig. 4.

Formation of Triol (II) from Epoxide (IV)—The stirred glacial acetic acid solution (15 ml) of 5,6 α -epoxy-5 α -cholestan-3 β -ol (IV) (60 mg, 1.49 × 10⁻⁴ mole) was mixed promptly with an aqueous solution (3 ml) of FeSO₄·7H₂O (276 mg) at 40° and 30% H₂O₂ (1.5 ml, *ca.* 1.5 × 10⁻² mole) was added to the mixture dropwise during 10 min. Sampling of the test solutions was made at the elapsed times of 10, 30, and 60 min from the first drop of H₂O₂. Each sample solution was worked up as described above and the ether extract obtained finally was submitted to GLC for the determination of II formed. The comparative reaction mixture lacking H₂O₂, FeSO₄, or both of them was also treated in the same procedure as described above. The rates of consuming the epoxide (IV) as a function of reaction time are shown in Fig. 5.

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