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Autoxidation of Cholesterol and Behavior of Its Hydroperoxide in Aqueous Medium[1]

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A model reaction was studied for the interaction between cholesterol (**1**) and molecular oxygen in biological matrices. An aqueous colloidal solution (60 ml) of **1** (2.7×10^{-4} mol) dispersed with sodium stearate (1.8×10^{-4} mol) was adjusted to pH 8 and energetically stirred at 85° without air-bubbling. Relative amounts of the sterols present in the reaction mixtures were determined every one hour by quantitative TLC and time-course of the oxyfunctionalization of **1** was followed for 9 hours. The major product in the early period was the 7-hydroperoxide (**2**) as an epimeric mixture of $\alpha:\beta \fallingdotseq 1:2$. The amounts of the 7-ketone (**3**) and 7-hydroxide (**4**) increased with decrease in that of **2** which was regarded as the intermediate. After passage of 7 hours the autoxidation system arrived at an apparent final state where consumption of **1** and formation of products were suspended and **2** was no longer detectable. More than 70% of **1** was likely to be consumed and the relative amounts of the oxygenated products (**3**, **4**, and the 5,6-epoxide) and the unchanged substrate (**1**) were in an approximate ratio of 45:25:5:25. The induction period was lost when a small amount (1×10^{-5} mol) of the 7α-hydroperoxide (**2a**) was initially present in the reaction mixture. From these results, the autoxidation of **1** under the conditions was assumed to have an ordinary pattern of the chain reaction initiated by the decomposition of hydroperoxide.

Keywords——time-course of oxyfunctionalization; decomposition of hydroperoxide; elimination of induction period; product-inhibition; quantitative TLC; GLC; intermediate; cholest-5-ene-7-hydroperoxides

Autoxidation of cholesterol has been the subject of a number of papers. In our previous study,[3] the same reaction was examined in the aqueous dispersions with four kinds of solubilizers and the reaction mixture dispersed with cholesteryl sulfate gave a new oxidation product, 5α-cholestane-3β,5,6β,7α-tetrol.[4] Bergström and Wintersteiner studied in detail this oxidation in aqueous colloidal solution and factors governing it.[5a,b] These authors proposed the intermediary formation of a hydroperoxide group on a methylenic carbon atom adjacent to the double bond. Smith *et al.* indicated that the autoxidation of cholesterol proceeded *via* initial formation of sterol hydroperoxide and the same substrate in the stearate dispersion afforded only the 7-hydroperoxides as initially formed products;[6a,b] the 5α-hydroperoxide failed to be detected.[6c] Recent progress in the studies on the lipid peroxidation and on the micellar effects in organic reactions prompted us to reinvestigate this reaction in aqueous media. In this study, the time-course of the oxyfunctionalization in cholesterol molecule was observed in the aerated aqueous colloidal solution dispersed with sodium stearate. To our knowledge, this is the first study that consumption of the substrate, formation as well

1) This paper constitutes Part IX of the series entitled "Metal Ion Catalyzed Oxidation of Steroids;" Part VIII: M. Kimura and T. Muto, *Chem. Pharm. Bull.* (Tokyo), **27**, 109 (1979).
2) Location: *Nishi-6-chome, Kita-12-jo, Kita-Ku, Sapporo, 060, Japan.*
3) M. Kimura, M. Kawata, and T. Sawaya, *Chem. Pharm. Bull.* (Tokyo), **24**, 2258 (1976).
4) M. Kawata, M. Tohma, T. Sawaya, and M. Kimura, *Chem. Pharm. Bull.* (Tokyo), **24**, 3109 (1976).
5) *a*) S. Bergström and O. Wintersteiner, *J. Biol. Chem.*, **141**, 597 (1941); *b*) *Idem, ibid.*, **145**, 309 (1942); *c*) *Idem, ibid.*, **145**, 327 (1942).
6) *a*) J.E. van Lier and L.L. Smith, *J. Org. Chem.*, **35**, 2627 (1970); *b*) L.L. Smith and F.L. Hill, *J. Chromatogr.*, **66**, 101 (1972); *c*) L.L. Smith, J.I. Teng, M.J. Kulig, and F.L. Hill, *J. Org. Chem.*, **38**, 1763 (1973).

as decomposition of the intermediate 7-hydroperoxides, and generation of the final products were simultaneously followed as a function of time during the autoxidation of cholesterol.

Results and Discussion

The aqueous stearate-dispersion of cholesterol (I) was adjusted to pH 8.0 with sodium hydroxide and efficiently stirred at 85° without air-bubbling, as reported.[7] Aliquots of the reaction mixture were taken out at varying times and the contents were submitted to thin-layer chromatography (TLC). The relative amounts of the oxidation products and the unchanged substrate were then determined by using the hydrogen flame ionization detector. The products identified were cholest-5-en-3β-ol-7-one (II), cholest-5-ene-3β,7α-diol (IIIa), cholest-5-ene-3β,7β-diol (IIIb), 3β-hydroxycholest-5-ene-7α-hydroperoxide (IVa), 3β-hydroxycholest-5-ene-7β-hydroperoxide (IVb), and 5,6-epoxycholestan-3β-ol (s) (V). The epimers of the products (III and IV) were derivatized to their trimethylsilyl ethers and determined by gas–liquid chromatography (GLC). The hydroperoxides (IV) were similarly estimated as the corresponding hydroxides (III) after being reduced with triphenylphosphine. Other oxidation products such as 5α-cholestane-3β,5,6β-triol, cholest-4-en-3-one, cholesta-3,5-diene, and cholesta-3,5-dien-7-one were also detectable in TLC, but negligible in amount.

The major products in the earlier period were the 7-hydroperoxides (IV) which gave maximum value after approximately three hours[8] and were the epimeric mixture in an approximate molar ratio of α(IVa): β(IVb)=1: 2. The amounts of the 7-ketone (II) as well as the 7-hydroxides (III) then increased rather rapidly with decrease in that of the hydroperoxides (IV). After approximately seven hours the autoxidation system arrived at an apparent final state where consumption of the substrate (I) as well as formation of the products were suspended and the hydroperoxides (IV) were no longer detectable (Fig. 1). The relative amounts of the oxidized sterols (II, III, and V) and the unchanged substrate (I) were estimated to be in an approximate ratio of 45: 25: 5: 25, indicating that seventy or more per cent of I was consumed. Epimers of the 7-hydroxide (III) were yielded in almost equal amount. Formation of the 7-ketone (II) and 7-hydroxides (III) in colloidal aqueous dispersion has been ascribed to the decomposition of 7α-hydroperoxide (IVa).[5b,6b] Self-reaction of the alkylperoxy radicals having α-hydrogen atom gives a mixture of the corresponding ketone and alcohol with generation of oxygen molecule.[9a,b] Smith *et al.* reported that neither direct attack of molecular oxygen (triplet or singlet) on I nor thermal decomposition of the common autoxidation products of I accounted for the formation of the 5,6-epoxides (V).[10] On the contrary, incubation of I with the 7-hydroperoxide (IV) under a nitrogen gas stream yielded a mixture of the epimeric epoxides (V).[10] Under our experimental conditions, the hydroperoxides (IV) initially formed may thus turn into the diols (III) by donating an oxygen atom to the intact substrate (I) which is then converted into the epoxides (V), probably through the homolytic pathways.[11] The preferential formation of the 7-ketone (II), that has also been observed in the prior studies,[5a,7,11,12] may considerably be contributed by the dehydration[5b] of the intermediates (IV), no oxidation of secondary alcohols such as I and III occurs under these conditions.[3,5b] The 5-hydroperoxide was undetectable as reported,[6c]

7) E.H. Mosbach, M. Nierenberg, and E.E. Kendall, *J. Am. Chem. Soc.*, **75**, 2358 (1953).
8) According to the study by Smith *et al.*,[6b] the time required for development of initial hydroperoxide was thirty minutes.
9) *a*) G.A. Russell, *J. Am. Chem. Soc.*, **79**, 3871 (1957); *b*) T. Minato, S. Yamabe, H. Fujimoto, and K. Fukui, *Bull. Chem. Soc. Jpn.*, **51**, 682 (1978).
10) L.L. Smith and M.J. Kulig, *Cancer Biochem. Biophys.*, **1**, 79 (1975).
11) Y. Ohkatsu and T. Tsuruta, *Bull. Chem. Soc. Jpn.*, **51**, 188 (1978); R.A. Budnik and J.K. Kochi, *J. Org. Chem.*, **41**, 1384 (1976).
12) N.D. Weiner, P. Noomnont, and A. Felmeister, *J. Lipid Res.*, **13**, 253 (1972).

while a slight portion of its specimen freshly prepared was found to be transformed into IV under our experimental conditions. Autoxidation of I in aqueous dispersion is unlikely to afford the 5-hydroperoxide which is known to be formed from I by excited-state (singlet) molecular oxygen.[13]

Similarly to the usual autoxidation reactions, an induction period was observed and its length varied considerably even under apparently identical conditions. The induction period was lost when a small amount of the 7α-hydroperoxide (IVa) was added to the reaction mixture (Fig. 2). The autoxidation of I was thus likely to have an ordinary pattern of the chain reaction initiated by the decomposition of hydroperoxide.

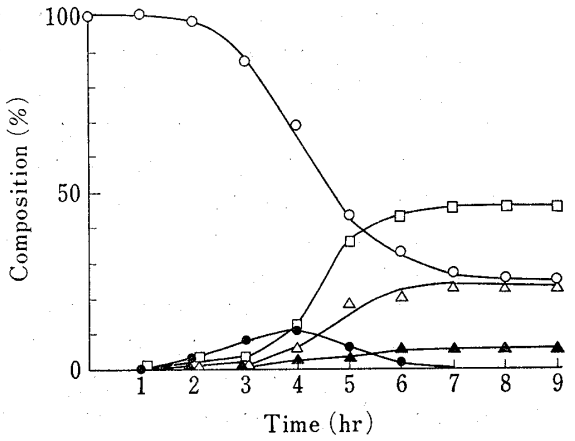

Fig. 1. Time Course of the Reaction

○, cholesterol (I).
□, 7-keto cholesterol (II).
△, 7-hydroxy cholesterol (III).
●, hydroperoxide (IV).
▲, epoxide (V).

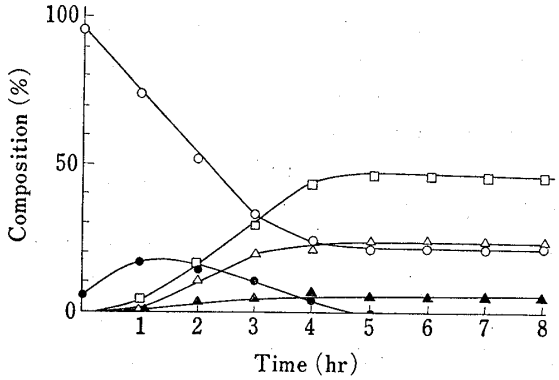

Fig. 2. Effect of Hydroperoxide initially added

○, cholesterol (I).
□, 7-ketocholesterol (II).
△, 7-hydroxy cholesterol (III).
●, hydroperoxide (IV).
▲, epoxide (V).

The autoxidation of I in aqueous media tends to arrive at an apparent final state, as shown in Figs. 1 and 2 and also observed in the prior studies.[5b,14] Such product-retardation was similarly observed in the dispersions with different detergents.[5b] It may be considered that these reactions involve an interaction-process of I with its oxygenated product. Alternatively, the autoxidation of I in aqueous dispersion may proceed differently from that in organic media. It is of interest in this respect that I was, in contrast to its esters, rather resistant to the oxidation when it was exposed to molecular oxygen by bubbling air through its refluxing or heating solution of organic solvent such as ethanol–ether,[15a] octane,[15b] benzene,[15c,d] toluene,[6b] or xylene.[15c] In colloidal aqueous solution, on the contrary, I was highly susceptible to the action of molecular oxygen,[5a,b,7,14,15a] while the esterification of I greatly diminished the susceptibility.[5c] Moreover, autoxidation of I did not occur when proteins such as bovine serum albumin, human whole plasma, and lyophylized human plasma were substituted for sodium stearate as dispersing agent.[14] The intermolecular interactions between I and lecithins which are surfactants of natural origin are strong and, particularly, dipalmitoyl lecithin which is a fully saturated phospholipid is very effective for inihibiting the autoxidation of I.[16] Such interaction with phospholipids has been postulated as a possible mechanism for the protection of membrane-bound I from oxidation.[16] These phenomena were considered

13) G.O. Schenk, *Angew. Chem.*, **69**, 579 (1957).
14) L.L. Smith, W.S. Matthews, J.C. Price, R.C. Bachmann, and B. Reynolds, *J. Chromatogr.*, **27**, 187 (1967).
15) *a*) L.N. Norcia, *J. Am. Oil Chem. Soc.*, **35**, 25 (1958); *b*) H.T. Tien, S. Garbone, and E.A. Dawidowicz, *Nature* (London), **212**, 718 (1966); *c*) A.H. Cook, *J. Chem. Soc.*, **1938**, 1774; *d*) L.F. Fieser, H.-Y. Huang, and B.K. Bhattacharyya, *J. Org. Chem.*, **22**, 1380 (1957).

to be physical rather than chemical in nature[16] and likely connected with the colloidal state of the systems.[5a] Further studies are in progress, focussing on the relationship between the oxygen susceptibility of cholesterol (I) and its molar ratios to the detergents in different concentrations.

Experimental

Materials and Authentic Specimens——Cholesterol (I), stearic acid, and triphenylphosphine were of commercial source and purified by repeated recrystallization from EtOH (cholesterol) and MeOH (the others). The authentic specimens of 3β-hydroxycholest-5-en-7-one (II, mp 164—166°; lit.[17a] mp 157°), cholest-5-ene-3β,7α-diol (IIIa, mp 174—175°; lit.[17b] mp 188—189°), cholest-5-ene-3β,7β-diol (IIIb, mp 172—175°; lit.[17b] mp 172—176°/180—181°), 3β-hydroxycholest-6-ene-5α-hydroperoxide (mp 147.5—149.8°; lit.[17c] mp 148—149°), 3β-hydroxycholest-5-ene-7α-hydroperoxide (IVa, mp 164—165°; lit.[17c] mp 154—155°), 5,6α-epoxy-5α-cholestan-3β-ol (Va, mp 143.5°; lit.[17d] mp 141—143°), 5,6β-epoxy-5β-cholestan-3β-ol (Vb, mp 130—133°; lit.[17e] mp 136°), 5α-cholestane-3β,5,6β-triol (mp 226—227°; lit.[17f] mp 237—239°), cholesta-3,5-diene (mp 78—79°; lit.[17g] mp 78.4—80.3°), cholesta-3,5-dien-7-one (mp 110—112°; lit.[17h] mp 111—112.5°), cholest-4-en-3-one (mp 78.5—80°; lit.[17i] mp 78.5—80.5°), and 5α-cholestane (mp 75—76°; lit.[17j] mp 78—79°) were prepared and purified as reported. Melting points were taken on a micro hot-stage apparatus and are uncorrected.

Autoxidation Reaction (Fig. 1)——After ethanol solution (2 ml) of stearic acid (50 mg, 1.8×10^{-4} mol) was mixed with deionized water (60 ml), the aqueous solution was adjusted to pH 8 with 0.1 N NaOH. Ethanol solution (3 ml) of cholesterol (I, 100 mg, 2.7×10^{-4} mol) was added to the aqueous stearate solution at 85° under vigorous stirring. In a flask open to the air and at 85±3°, the aqueous colloidal dispersion obtained was energetically stirred further by a blade (diameter 3 cm, 20±5 rps), with occadional supply of water in order to maintain the initial volume.

Reaction initiated with Hydroperoxide (Fig. 2)——To an aqueous stearate solution mentioned above, ethanol solution (3 ml) of I (100 mg, 2.7×10^{-4} mol) and the 7α-hydroperoxide (IVa, 5 mg, 1×10^{-5} mol) was added at 85° under vigorous stirring. The aqueous colloidal dispersion obtained was then similarly treated as described above.

Thin-Layer Chromatography——TLC was carried out on silica gel (Merck 60F254, 200 μm thick) plate with the solvent system of benzene–EtOAc (3:2). The plate was then dried in air and stained by spraying 50% H_2SO_4 with subsequent heating on a hot-plate for several minutes. Hydroperoxide was visualized (red) with the N,N-dimethyl-*p*-phenylenediamine reagent.[6d] *Rf* value (color after heating or UV-254 nm-absorption before spraying): cholesta-3,5-diene, 0.95 (red); cholesta-3,5-dien-7-one, 0.94 (UV): cholest-4-en-3-one, 0.89 (UV); I, 0.75 (red); V, 0.41 (yellow); IV (IVa+IVb), 0.38 (bl.green); II, 0.35 (UV); IIIb, 0.27 (blue); IIIa, 0.21 (blue); cholestane-3β,5α,6β-triol, 0.05 (yellow).

Preparative TLC was carried out on silica gel (Wako gel B-5F, 0.75 mm thick) plate (20×20 cm) and developed with the same solvent system mentioned above.

Gas-Liquid Chromatography——GLC was carried out by using a Shimadzu GC-3BF gas chromatograph equipped with a hydrogen flame ionization detector and the glass tube (3 mmϕ×2 m) packed with 2% OV-225 on Shimalite W (80—100 mesh); conditions: N_2 as carrier gas (80 ml/min), column temperature at 230°, detector temp. at 250°, 5α-cholestane as an internal standard.

A portion of the reaction mixture was taken out at the elapsed time of 3 hr and its ether extract was submitted to a preparative TLC. The respective chromato-zone containing the diols (III) or the hydroperoxides (IV) was separated and extracted with $CHCl_3$. The organic layer was evaporated *in vacuo* to give the residue which was submitted to GLC as a trimethylsilyl ether.

To a pyridine solution (0.2 ml) of the hydroxide (III) fraction (3 mg) and 5α-cholestane (5 mg) were added N,O-bis(trimethylsilyl)acetamide (0.1 ml) and a drop of trimethylchlorosilane (Fr. 1). The hydroperoxide (IV) fraction (5 mg) and 5α-cholestane (5 mg) were dissolved in pyridine (0.2 ml) and triphenyl-

16) N. Weiner, W.C. Bruning, and A. Felmeister, *J. Pharm. Sci.*, **62**, 1202 (1973).

17) *a*) K. Heusler and A. Weststein, *Helv. Chim. Acta*, **35**, 284 (1952); *b*) C.W. Shoppee and B.C. Newman, *J. Chem. Soc.* (*C*), **1968**, 981; *c*) G.O. Schenck, O.A. Neumüller, and W. Fisfeld, *Ann. Chem.*, **618**, 202 (1958); *d*) L.F. Fieser and M. Fieser, "Reagents for Organic Synthesis," John Wiley and Sons, Inc., London, 1967, p. 136; *e*) J. Hattori, *Yakugaku Zasshi*, **60**, 125 (1940); S.G. Levine and M.E. Wall, *J. Am. Chem. Soc.*, **81**, 2826 (1959); *f*) L.F. Fieser and S. Rajagopalan, *J. Am. Chem. Soc.*, **71**, 3938 (1949); *g*) F.C. Chang and N.F. Wood, *Steroids*, **4**, 55 (1964); *h*) A. Nickon and J.F. Bagli, *J. Am. Chem. Soc.*, **83**, 1498 (1961); *i*) R.V. Oppenauer, "Organic Syntheses," Coll. Vol. 3, ed. by E.C. Horning, John Wiley and Sons, Inc., New York, 1955, p. 207; *j*) S. Yamamura, M. Toda, and Y. Hirata, *ibid.*, Vol. 53, ed. by A. Brossi, 1973, p. 86.

phosphine (20 mg) was added to the solution, in order to reduce IV into III. The reaction mixture was then trimethylsilylated similarly as described above (Fr. 2).

t_R(min): triphenylphosphine (4.8), 5α-cholestane (11.4), 7α-hydroxycholesterol trimethylsilyl ether (18.6), 7β-hydroxycholesterol trimethylsilyl ether (29.0), triphenylphosphine oxide (43.4). Peak area: 7α-hydroxycholesterol (IIIa)≧7β-epimer (IIIb) in Fr. 1; 7α-hydroperoxide (IVa): 7β-epimer (IVb)≒1:2 in Fr. 2.

Determination of Product Distribution——Aliquot of the reaction mixture was taken out every one hour and extracted with $CHCl_3$. The organic layer was worked up as usual to give the residue, thin-layer chromatogram of which was recorded on an IATRON Model TFG-10 Thinchrograph using the solvent system of cyclohexane–EtOAc (3:1) with duplicate development.

The sum of the peak areas shown by cholesta-3,5-diene, cholesta-3,5-dien-7-one, and cholest-4-en-3-one comprised *ca.* 2% of the total peak areas on the chromatogram. Cholestane-3β,5α,6β-triol was similarly estimated to be of *ca.* 2.5%. In preparation of the time-course diagrams (Fig. 1, 2) these minor products were neglected for convenience and the compositions (%) were calculated on the basis of the peak areas given by I, II, III, IV, and V as major components.

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