10. Peng, S.-K., and C.B. Taylor, in *Dietary Fats and Health*, Monograph No. 10, Chapt. 57, edited by E.G. Perkins and W.J. Visek, American Oil Chemists' Society, Champaign, IL (1983).
11. Finocchiaro, E.T., and T. Richardson, *J. Food Prot. 46*:917 (1983).
12. Tsai, L.-S., K. Ijichi, C.A. Hudson and J.J. Meehan, *Lipids 15*:124 (1980).
13. Tsai, L.-S., and C.A. Hudson, *J. Fd. Sci. 49*:1245 (1984).
14. Tsai, L.-S., and C.A. Hudson, *Ibid. 50*:229 (1985).
15. Herian, A.M., and K. Lee, *Ibid. 50*:276 (1985).
16. Missler, S.R., B.A. Wasilchuk and C. Meritt Jr., *Ibid. 50*:595 (1985).
17. Naber, E.C., and M.D. Biggert, *Poult. Sci. 64*:341 (1985).
18. Finocchiaro, E.T., K. Lee and T. Richardson, *J. Amer. Oil Chem. Soc. 61*:877 (1984).
19. Ryan, T.C., J.I. Gray and I.D. Morton, *J. Sci. Food Agric. 32*:305 (1981).
20. Highley, N.A., S.L. Taylor, A.M. Herian and K. Lee, *Meat Sci. 16*:175 (1986).
21. Fieser, L.F., *Org. Synth. Col. 4*:195 (1963).
22. Fieser, L.F., and S. Rajagopalan, *J. Am. Chem. Soc. 71*:3938 (1949).
23. Davis, M., and V. Petrow, *J. Chem. Soc.* 2536 (1949).
24. Chicoye, E., W.D. Powrie and O. Fennema, *Lipids 3*:335 (1968).
25. Flanagan, V.P., A. Ferreti, D.P. Schwartz and J.M. Ruth, *J. Lipid Res. 16*:97 (1975).
26. Williams, L.D., and A.M. Pearson, *J. Ag. Food Chem. 13*:573 (1965).
27. Chicoye, E., W.E. Powrie and O. Fennema, *J. Food Sci. 33*:581 (1968).
28. Maxwell, R.J., and D.P. Schwartz, *J. Amer. Oil Chem. Soc. 56*:634 (1979).
29. Association of Official Analytical Chemists, *Official Methods of Analysis*, 14th ed., 1984, Method 28:092.
30. Bascoul, J., N. Domergue and A. Crastes de Paulet, *J. Steroid Biochem. 19*:1779 (1983).
31. Shen, C.-S.J., and A.J. Sheppard, *Chromatographia 17*:469 (1983).
32. Ansari, G.A.S., and L.L. Smith, *J. Chromatog. 175*:307 (1979).
33. Csiky, I., *J. Chromatog. 241*:381 (1982).
34. Tsai, L.-S., and C.A. Hudson, *J. Amer. Oil Chem. Soc. 58*:931 (1981).
35. Fioriti, J.A., and R.J. Sims, *Ibid. 44*:221 (1967).
36. Korahani, V., J. Bascoul and A. Crastes de Paulet, *J. Chromatog. 211*:392 (1981).
37. Krull, V.J., M. Thompson and A. Arya, *Talanta 31*:489 (1984).
38. Teng, J.I., M.J. Kulig and L.L. Smith, *J. Chromatog. 75*:108 (1973).
39. Gumulka, J., J. St. Pyrek and L.L. Smith, *Lipids 17*:197 (1982).

[Received September 23, 1985]

# Cholesterol Oxides II. Measurement of the 5,6-Epoxides During Cholesterol Oxidation in Aqueous Dispersions

**G. Maerker** and **F.J. Bunick**

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118

**Cholesterol in aqueous dispersion with sodium stearate or Triton surfactants was aerated at various pH values at 50 and 80 C. Analysis of the reaction mixtures by TLC during the oxidation produced qualitatively similar patterns regardless of pH or temperature. Major oxidation products observed were 7-ketocholesterol, the isomeric 7-hydroperoxy- and 7-hydroxycholesterols, the isomeric 5,6-epoxycholestanols and 3β,5α,6β-trihydroxycholestanol. The concentrations of 3β,5α,6β-trihydroxycholestanol and an unknown compound increased greatly at the lower pH values.**

**Recovery of the 5,6-epoxide isomers by preparative TLC followed by capillary GC allowed the α- and β-epoxide isomers to be quantitated. Oxidations at pH 8 and 12 produced increasing amounts of the epoxides with time, without significant changes in the α/β-epoxide ratio. However, oxidations at the acidic pH values of 5.5 and 3 showed large changes in the α/β-epoxide ratio during the oxidation. Measurement of the hydrolysis rates of the 5,6-epoxides at pH 5.5 showed that the β-epoxide isomer is more labile than the α-isomer by a factor of 2.5. The rate constant for the hydrolysis of the α-epoxide isomer was 5.3 × $10^{-7}$ $sec^{-1}$ and that of the β-isomer 13 × $10^{-7}$ $sec^{-1}$.**

The autoxidation of cholesterol has been reported for many years by a number of researchers using a variety of experimental conditions (1-9), and a large number of oxidation products have been identified (10-16). Reports that several of the common cholesterol oxidation products adversely affect biological systems have increased interest in this area, especially with regard to the initiation of atherosclerosis (17-21).

Among the oxidation products cited as having detrimental biological effects are the 5α,6α-epoxide (22-28) and its hydration product 3β,5α,6β-trihydroxycholestanol (29-34). A number of studies have reported that the isomeric cholesterol epoxides are formed in relatively fixed ratios, the α/β ratio being a function of reaction conditions (35-40). Ratios reported have varied from 1:11 in favor of 5β,6β-epoxycholestanol to 8:1 in favor of the α-isomer. However, these ratios have for the most part been reported as single point analyses. We wanted to determine whether the α/β epoxide ratio was affected by factors other than stereoselectivity during formation.

In contrast to previous studies, the present work measures the α/β-epoxide ratios throughout the course of cholesterol oxidation. Epoxide quantitation and ratio measurement were greatly facilitated by use of capillary gas chromatography on underivatized cholesterol oxide isolates (36,41). Autoxidation of cholesterol in aqueous dispersion was chosen because rapid oxidation could be achieved with aeration at relatively low temperatures (50-80 C). In addition, colloidally dispersed cholesterol may be a better model for the state of cholesterol in foods and in the aqueous fluids of animal tissues than cholesterol dissolved in organic solvents or in the crystalline state (5,15). Moreover, the buffered aqueous

medium used in this study allowed a systematic examination of the effects of pH along with temperature on the $\alpha/\beta$ epoxide ratio during cholesterol oxidation by air.

## EXPERIMENTAL PROCEDURES

*Materials.* Cholesterol, ash free and precipitated from alcohol, was purchased from Sigma Chemical Company (St. Louis, Missouri). Cholesterol oxide standards were purchased from Sigma Chemical Company (St. Louis, Missouri); Steraloids, Inc. (Wilton, New Hampshire) and Research Plus, Inc. (Bayonne, New Jersey). Cholestanol-5$\beta$,6$\beta$-epoxide was prepared from cholesterol via 3$\beta$,5$\alpha$,6$\beta$-trihydroxycholestanol (50) and the corresponding triacetate (51) by the method of Chicoye et al. (14). The $\beta$-epoxide was purified by preparatory TLC.

Triton X-100, Triton X-102, Triton X-114 and Triton QS-30 were obtained from Rohm and Haas Company (Philadelphia, Pennsylvania); Tween 80 from ICI Americas, Inc. (Wilmington, Delaware); Tergitol 1559 and SAG 47 Silicone Antifoam Fluid from Union Carbide Corp. (Moorestown, New Jersey); stearic acid (technical grade) was purified by double recrystallization.

TLC plates, silica gel G and GHL (250 microns), were purchased from Analtech (Newark, Delaware); compressed air, zero grade, from Air Products & Chemicals, Inc. (Tamaqua, Pennsylvania).

All solvents used were "distilled in glass grade," and chemicals were of reagent grade quality. Water was double deionized, glass distilled.

*I. Oxidation of cholesterol.* (i) Dispersion media. To 150 ml of distilled, deionized water containing 50 mg of disodium phosphate was added either 150 mg of Triton X-100 or 125 mg of stearic acid as surfactant. Triton X-100 was used at pH values of 8 or below and stearic acid at pH 8 or above. At pH 3, a mixture of equal amounts of Triton X-100 and Triton QS-30 was found to be considerably more effective in dispersing cholesterol than Triton X-100 alone.

For stearate containing media, stearic acid dissolved in 4 ml of ethanol was added dropwise to water at 80 C with vigorous stirring. To the resulting turbid suspension, 1 *N* NaOH was added dropwise until the solution clarified, at which point it was cooled to room temperature. The pH of the stearate or Triton solutions was measured, adjusted to pH 8 and the solution stored at room temperature until used. Adjustments in the pH of the dispersion medium to values other than 8 were made after addition of the cholesterol.

(ii) Apparatus. The reaction vessel consisted of a four-neck 250 ml round bottom flask fitted with a water-cooled condenser, thermometer and gas dispersion tube. Temperature was maintained at $\pm 1.0$ C with an $I^2R$ Thermo-O-Watch controller connected to a rheostated heating mantle. Air was supplied from a compressed air tank at 200 cc/min via an Ascarite II trap to remove $CO_2$. The reaction vessel was placed on a magnetic stirrer inside a light proof cabinet during use.

(iii) Cholesterol autoxidation. Cholesterol (75 mg) dissolved in 2 ml of ethanol was injected slowly into 150 ml of dispersion media being vigorously stirred and heated at 80 C in a 250 ml beaker on a hot plate. After cooling, the pH of the solution was adjusted to the desired value, and samples were removed as the unoxidized controls. The mixture was reheated to reaction temperature and transferred to the reaction vessel, 3 drops of antifoaming agent were added, and the flow of air through the mixture was initiated. Samples were removed periodically for analysis. Typically, two 5 ml-aliquots were taken at each sampling for separate analyses by GC or TLC. The aliquots were cooled, their pH measured and adjusted to 9, and then they were stored in 24 ml screw capped vials under nitrogen at $-20$ C until analyzed.

(iv) Product isolation. Frozen samples were thawed and internal standard (5$\alpha$-cholestan-3$\beta$-ol-6-one) was added to samples to be analyzed by GC. Reaction products were extracted from both sets of samples with two 10 ml and one 5 ml aliquot of ethyl acetate per sample. Sodium sulfate was used to break emulsions. Extracts of each sample were pooled, dried over anhydrous sodium sulfate, and solvent removed under a stream of nitrogen.

Samples were reconstituted with ethyl acetate using 0.5 ml for analytical TLC samples and 0.1 ml for GC samples. Analytical TLC samples were used as such, while GC samples were fractionated by preparative TLC prior to gas chromatography as follows: 20 $\mu$l of the 0.1 ml GC sample was streaked onto each of two 5 $\times$ 20 cm TLC plates and developed in benzene-ethyl acetate (3:2, v/v). One plate was charred to locate the 5,6-epoxide band. This band was scraped from the second plate onto weighing paper and transferred to a 10 cc syringe fitted with a 0.45 $\mu$m Millipore filter. Two successive 4 ml extractions with acetone were used to elute products into a 9 ml screw capped vial. Acetone was removed with a stream of nitrogen, and samples were reconstituted with 0.1 ml of ethyl acetate and stored at $-20$ C prior to GC analysis.

(v) Analytical thin layer chromatography. Prior to use, plates were washed by development with chloroform-methanol (2:1, v/v) and activated overnight in a 115 C air oven. Plates containing samples were developed with benzene-ethyl acetate-acetic acid (60:40:1, v/v/v). Air dried plates were sprayed lightly with 50% sulfuric acid, placed on an unheated hot plate and gradually warmed to produce a maximum color display of the cholesterol oxidation products. After complete charring at 220 C, the plates were removed from the hot plate, cooled, and $R_f$ values were measured.

Specific oxidation products were identified by comparison of mobilities with those of authentic compounds and by color development during charring. In addition, hydroperoxides were identified by use of a Wurster dye (42) and by sodium borohydride reduction combined with 2-dimensional TLC; ketones by reaction with 2,4-dinitrophenylhydrazine (43) and UV absorption; 7$\alpha$- and 7$\beta$-hydroxy isomers by recovery of bands from TLC plates followed by determination of GC retention times of the extracted compounds; 5,6-epoxides by recovery of TLC band followed by GC with and without hydrobromination of the sample.

During the authentication of the 5$\beta$,6$\beta$-epoxycholestanol synthesized for this study, the observation was made that the $\beta$-epoxide gives an initial blue color response with sulfuric acid on a warm TLC plate. After a short period of heating, the blue color changes to a yellowish tan typical of the $\alpha$-epoxide isomer which does not elicit the initial blue coloration. Previously, 5,6-epoxides were reported to give only a yellow color.

(vi) Gas liquid chromatograph. Underivatized samples

prepared for GC were chromatographed on a Sigma 2000 capillary chromatograph (Perkin-Elmer) equipped with a SGE-OCI2 on-column injector and fitted with a 0.2 mm I.D. × 25 crosslinked SE-54 (film thickness 0.33 μm) FSOT (Hewlett Packard Ultra #2) column having a 0.32 mm I.D. × 2 m deactivated fused silica retention gap. Carrier gas was helium at a flow rate of 36 cm/sec. Temperature program was as follows: Isothermal at 100 C for 5 min followed by a 30 C/min increase to 260 C followed by a 0.5 C/min increase to 300 C. Detection was by F.I.D., and peak areas were integrated with an LCI-100 recording integrator (Perkin-Elmer). Further details of the GC procedure are in Ref. 41.

(vii) Determination of α/β epoxide ratios. A standard solution of the two epoxides was prepared in ethyl acetate, so that 1 μl of solution contained approximately 70 ng of each isomer. The solution was injected in the gas chromatograph four times. The mean α/β ratio ± S.D. was 0.90 ± 0.02. The same solution was applied to three TLC plates to study recovery. Since the α-epoxide runs slightly behind the β-isomer on TLC it was necessary to be generous in scraping the area below the epoxide band. Triplicate recovery from TLC gave a mean α/β ratio ± S.D. of 0.90 ± 0.11.

(viii) Cholesterol quantitation. GC samples (see Product Isolation) prepared for analysis of oxidation products were also used for cholesterol determination but without prior fractionation. An aliquot of sample was diluted 500 times in ethyl acetate and 1 μl analyzed by gas chromatography. Peak areas were compared and quantities assigned based on the amount of cholesterol present in the unoxidized cholesterol dispersion.

*II. Hydrolysis of the 5,6-epoxide isomers.* (i) Reaction mixture. Epoxide hydrolysis was conducted in 100 ml of a Triton X-100 dispersion medium buffered with citrate (25.8 mg citric acid + 198.5 mg sodium citrate per 100 ml of dispersion medium). The pH was adjusted to 5.5 by the addition of a concentrated citric acid solution. Subsequently, a mixture containing 1 mg each of 5α,6α-epoxycholestanol, 5β,6β-epoxycholestanol and 5α-cholestan-3β-ol-6-one (internal standard) was dissolved in 1 ml of ethanol and transferred quantitatively to the dispersion medium at 60 C. This reaction mixture subsequently was transferred to the reaction vessel previously described but without the gas dispersion tube. The head space of the reaction vessel was purged with $N_2$, and the mixture was heated to 80 C. Samples (1 ml) taken periodically received a 0.08 ml addition of 0.1 *N* NaOH to raise their pH to about 9, thereby stabilizing the epoxides.

(ii) Substrate isolation. Cholesterol oxides were extracted from the samples with two 1 ml aliquots of ethyl acetate. Extracts were pooled, dried over anhydrous sodium sulfate and loaded onto an ethyl acetate-washed silica Sep-Pak (Waters Associates) to remove extracted Triton X-100 which interferes with GC analysis. Eluate from sample addition was discarded, and oxides were eluted with 1 ml of ethyl acetate plus drainage of the Sep-Pak. Sample without further preparation was analyzed by gas chromatography and the unreacted epoxides quantitated.

## RESULTS AND DISCUSSION

The facility with which cholesterol is oxidized by air in an aqueous medium depends in part on its degree of dispersion. Previous workers have employed sodium stearate as dispersing agent in alkaline or neutral medium, and we have followed their example. Other researchers have reported that cholesterol in aqueous dispersion with sodium stearate (1-3,11) oxidizes poorly at acidic pH values and at mild temperature (37-50 C) even in the presence of copper (II) ion as catalyst. This is reasonable, because at low pH values sodium stearate is converted to the free acid which has a melting point of 70.1 C, is insoluble in water and hence is a poor dispersing agent. Several nonionic surfactants were tested for their ability to disperse cholesterol in acidic, aqueous medium. These surfactants also were examined by TLC to determine relative purity and whether their chromatographic patterns would interfere with the detection of the cholesterol oxidation products. Triton X-100 and Triton X-102 proved to be chromatographically cleaner (interferences only near origin) than Tween 80, Tergitol 1559 and Triton X-114 and hence were used. In addition, both Triton X-102 and Triton Qs-30, an anionic phosphate surfactant used in combination with Triton X-102 at pH 3, proved stable in aqueous solution after 96 hr at 80 C with an air flow of 200 cc/min.

TLC proved useful in assessing the oxidizability of cholesterol dispersions made with various surfactants and buffered to different pH values. The decrease in size of the cholesterol spot with time of oxidation, as well as the presence of oxidation products, allowed a qualitative assessment of the relative equivalence of two dispersions in terms of the amount of cholesterol oxidized within a given time. Table 2 presents the relative percent of

TABLE 1

**Identification of the Major Cholesterol Oxidation Products Seen in Figure 1. Structures Are Shown in Figure 5**

| $R_f$ | Compound |
|---|---|
| 0.69 | Dispersing agent (stearic acid) |
| 0.55 | Cholesterol |
| 0.40 | 7β-Hydroperoxycholesterol |
| 0.37 | 7α-Hydroperoxycholesterol |
| 0.31 | 5,6-Epoxycholestanol + 7-ketocholesterol |
| 0.21 | 7β-Hydroxycholesterol |
| 0.17 | 7α-Hydroxycholesterol |
| 0.05 | 3β,5α,6β-Trihydroxycholestanol |

TABLE 2

**Percent of Cholesterol Oxidized in 24 Hours at 80 C. Effect of Dispersants**

| pH | Sodium stearate | Triton X-100 | Triton X-102 + Triton QS-30 (1:1) |
|---|---|---|---|
| 12 | 38 | <1 | nd[b] |
| 8 | 48 | 42 | nd |
| 5.5 | nd | 40[a] | nd |
| 3 | nd | <2 | 11 (55[a]) |

[a]After 96 hr.

[b]nd, not determined.

cholesterol oxidized in aqueous dispersions using different surfactants at four pH values. Values for percent cholesterol oxidized were determined by GC analysis. As seen, dispersions made with sodium stearate allowed significant amounts of the cholesterol to oxidize after only 24 hr at 80 C at alkaline pH values. Triton X-100 proved effective for dispersing cholesterol at intermediate pH values but not at pH extremes. The poor oxidizability of cholesterol dispersed in water with Triton X-100 at pH 12 and 3 is probably a result of a physical rather than a chemical phenomenon. At these pH values, the dispersions were much less translucent than at pH 5.5 and 8. Dispersions which produced significant cholesterol oxidation exhibited the bluish-gray color of a colloidal suspension, whereas the whiter appearance of those dispersions which did not readily oxidize indicate that the particle size of cholesterol was much larger. At pH 3, the addition of Triton QS-30, along with Triton X-100, dispersed the cholesterol well enough to allow for good oxidation when the reaction time was extended.

Cholesterol oxidized by air in aqueous dispersions was examined by TLC to identify the major products of oxidation and to assess the oxidizability of cholesterol in dispersions at different pH values. A typical TLC chromatogram is shown in Figure 1 for a pH 8 cholesterol didspersion oxidized at 80 C. Equal volumes of sample were applied to the TLC plate in order of increasing time of oxidation. Cholesterol oxidation products are listed by $R_f$ values in Table 1.

In the unoxidized control sample (0 hr), cholesterol chromatographs at $R_f$ 0.55 with trace amounts of endogenous oxides visible at lower $R_f$ values. Stearic acid, the dispersing agent used for this particular oxidation, is seen at $R_f$ 0.69. The addition of 1% glacial acetic acid to the solvent system is required to prevent streaking of the stearic acid on the TLC plate. In the experiment shown in Figure 1, cholesterol decreased by 48% after 24 hr, and oxidation products increased with time except in the case of the hydroperoxides which reached a maximum at 9 hr and subsequently decreased. Other researchers have shown that the 7-hydroperoxides are the initial oxidation products which react further to give the 7-ketone, the isomeric 7-hydroxides and 5,6-epoxides (15,45,47). The decomposition of hydroperoxides to secondary oxidation products is typical of autoxidations of lipids and has been observed in a number of instances (44,45). The observation that 7$\beta$-hydroperoxycholesterol was produced more abundantly than the $\alpha$-isomer is also consistent with observations by other researchers (15,45–48). The triol seen at $R_f$ 0.05 is a secondary reaction product resulting from the hydration of the 5,6-epoxides which co-chromatograph at $R_f$ 0.31 with 7-ketocholesterol, the major oxidation product formed in these oxidations. Oxidations of aqueous dispersions at other pH and temperature conditions produced similar TLC patterns with the notable exception that at lower pH values much larger quantities of triol were observed while at pH 12 almost no triol was detected. Also, at pH 3, a spot visible in Figure 1 between $R_f$ 0.21 and 0.31 became prominent but has not yet been identified. Among the various products formed during cholesterol autoxidation are the isomeric 5,6-epoxides whose relative ratios have been cited as being variable depending on the conditions of oxidation (35,36). The effects of pH and temperature on the ratio and quantity of epoxides were examined.

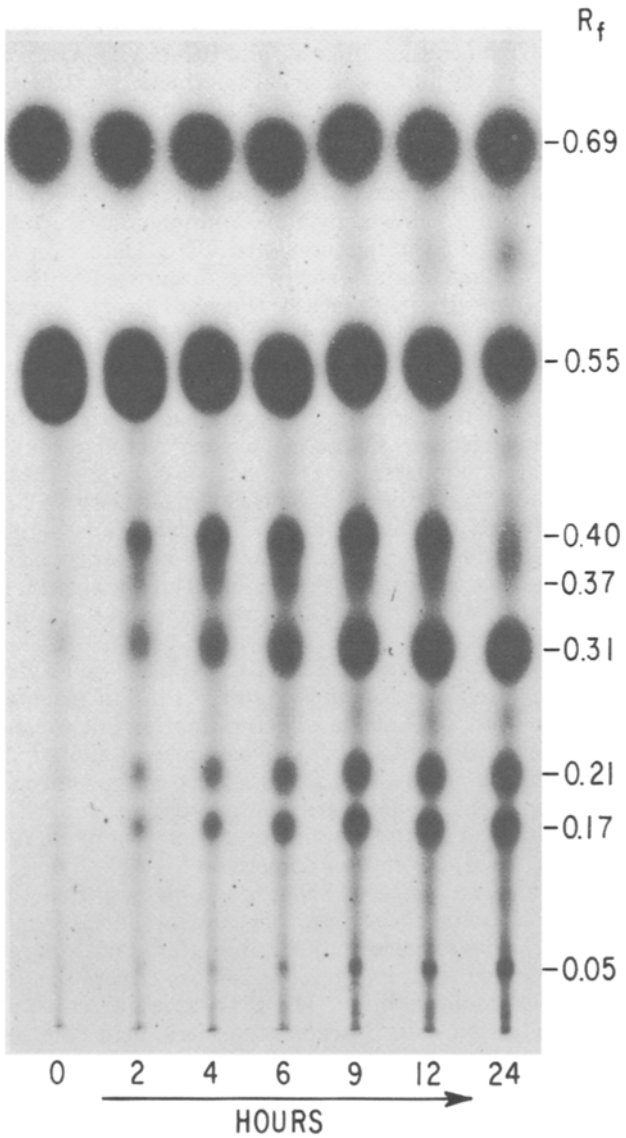


**FIG. 1. Thin layer profile of cholesterol autoxidation products from an aerated aqueous dispersion of cholesterol at pH 8, 80 C. Major products are identified in Table 1 by $R_f$ values**

Quantitative results from cholesterol oxidations conducted at four different pH values are shown in Figure 2. The temperature was 80 C in all four reactions. At pH 12, the epoxides are seen to increase with time during the first 24 hr after which only a small increase is observed. At pH 8 the epoxides also increase with time; however, their amounts are considerably greater than at pH 12. Similarly, at pH 5.5, the amounts of the epoxides increase with time. At this pH, though, a distinct maximum occurs at 72 hr beyond which the quantity of total epoxides decreases sharply. Such a maximum is indicative of competing reactions involving the formation and decomposition of the epoxides. At pH 3, the epoxides do not increase despite the fact that 55% of the initial cholesterol is oxidized after 96 hr.

The formation of the epoxides was coincident with the presence of cholesterol 7-hydroperoxide. At pH 12, for ex-

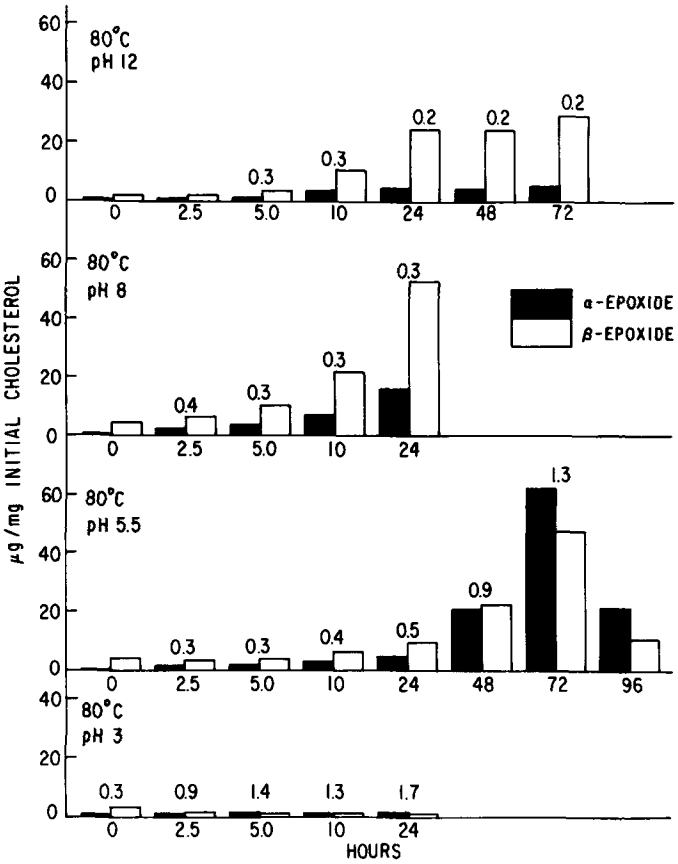


FIG. 2. Quantitation of the 5,6-epoxide isomers during oxidation of cholesterol at 80 C in aqueous dispersion at pH 12, 8, 5.5 and 3. The $\alpha/\beta$-epoxide ratio is given as the superscript above the bars.

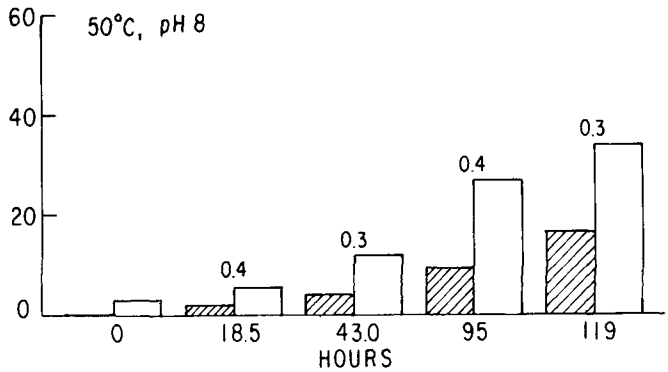


FIG. 3. Quantitation of the 5,6-epoxide isomers during oxidation of cholesterol at 50 C in aqueous dispersion at pH 8. The $\alpha/\beta$-epoxide ratio is given as the superscript above the bars.

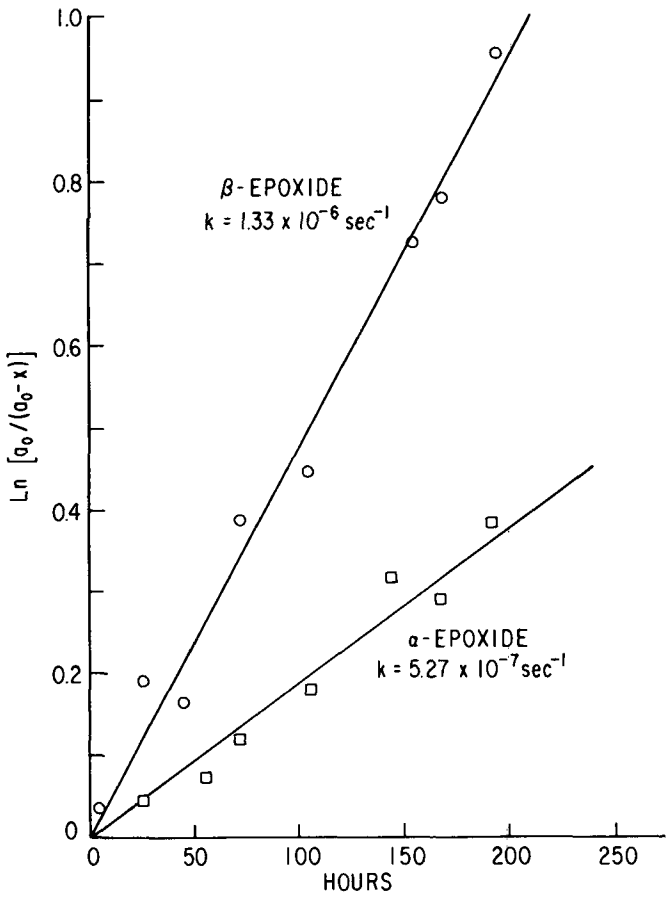


FIG. 4. First order rate plot for the hydrolysis of the $\alpha$- and $\beta$-epoxide isomers at pH 5.5, 80 C.

ample, hydroperoxides were observed to increase in concentration up to 24 hr in parallel with the 5,6-epoxides. After 48 hr, however, the levels of hydroperoxides were low and little or no further increase in the epoxides had occurred. Likewise at pH 8 and 5.5, maximum concentrations of hydroperoxides were observed in samples yielding the highest total epoxide amounts. This trend was not followed at pH 3. Although hydroperoxides were detected in amounts similar to those found at the higher pH values, the epoxides did not build up, presumably due to acid-catalyzed hydration of the epoxides to 3$\beta$,5$\alpha$,6$\beta$-trihydroxycholestanol. These results are consistent with the epoxidation of cholesterol by cholesterol hydroperoxide during the autoxidation reactions.

The measured ratio of the $\alpha$-epoxide to its $\beta$-isomer ($\alpha/\beta$) is shown in Figure 2 as superscripts above the bars. At pH 12 and 8 the $\alpha/\beta$ epoxide ratio is constant within the limits of experimental error and suggests that the $\alpha$- and $\beta$-isomers are formed at constant rates throughout these oxidations. Stereoselectivity clearly favors the $\beta$-epoxide isomer in these aqueous autoxidations. At pH 5.5, however, the $\alpha/\beta$ epoxide ratio is no longer constant throughout the reaction but changes considerably from an initial value of 0.3 to 2.1 after 96 hr. The maximum in the amount of total epoxide observed at 72 hr indicates that both epoxides are labile at pH 5.5. Furthermore, the changing $\alpha/\beta$ ratio indicates that there is a differential lability between the $\alpha$- and $\beta$-isomers. Stereoselectivity in epoxide formation is unlikely to account for this changing ratio, since it has been reported that the 7$\alpha$- and 7$\beta$-hydroperoxycholesterol isomers, assumed to be the major epoxidizing agents in this system, each epoxidize cholesterol to the same $\alpha/\beta$-epoxide ratio (37).

At pH 3, acid-catalyzed hydrolysis is fast enough to prevent any sizable buildup of the epoxides. However, the small amount of detectable epoxides remaining clearly show a change in the $\alpha/\beta$ epoxide ratio. As at pH 5.5, it is clear that the $\alpha$-epoxide is less sensitive to acid catalyzed hydration than the $\beta$-epoxide. The effect of

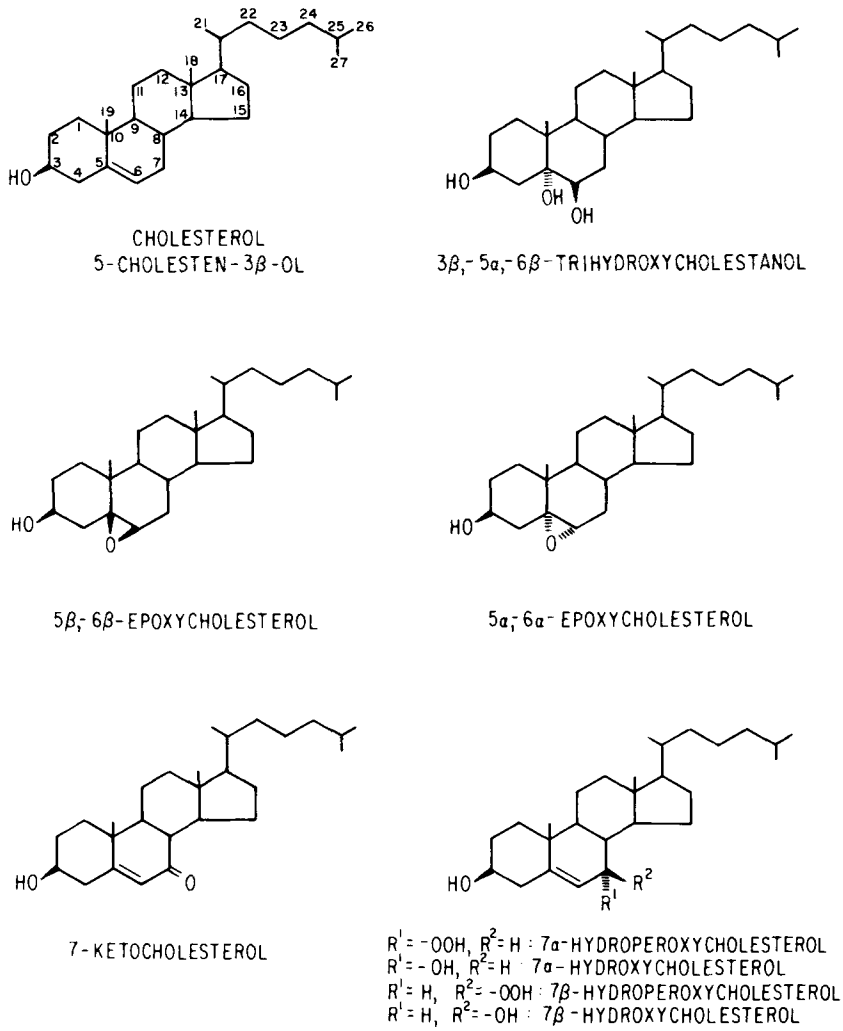


**FIG. 5. Structures of cholesterol and relevant oxidation products.**

temperature was examined by comparing cholesterol autoxidations at 50 C and 80 C at pH 8. The results of the epoxide analysis for the 50 C reaction are presented in Figure 3 and show a very similar pattern to the 80 C reaction of Figure 2. The only observed difference between the 50 C and 80 C oxidations is the expected longer time required for the reaction to proceed at the lower temperature.

An aqueous dispersion of cholesteryl stearate was autoxidized at 80 C, pH 8 to see whether the presence of a long saturated sidechain would affect the $\alpha/\beta$-epoxide ratio via steric hindrance. Analyses of the reaction mixture after 22.5 hr by TLC following mild saponification (49) of the cholesteryl ester revealed only the typical oxidation products as seen in Figure 1 for cholesterol oxidized at 80 C, pH 8. Furthermore, the measured $\alpha/\beta$ epoxide ratio of 0.3 was also the same as that found for oxidized free cholesterol under the same reaction conditions.

To assess the stability of the 5,6-epoxide isomers toward acid catalyzed hydrolysis, the authentic epoxide isomers were incubated at 80 C in an aqueous dispersion at pH 5.5 and the mixture assayed periodically for the amount of each epoxide remaining.

Quantitative differences between the two epoxide isomers are evident in the first-order rate plots of the data from the hydrolysis experiment (Fig. 4). The slopes of the lines in this plot yield rate constants of $13 \times 10^{-7}$ $sec^{-1}$ for the $\beta$-epoxide and $5.3 \times 10^{-7}$ $sec^{-1}$ for the $\alpha$-epoxide indicating that the $\beta$-isomer is about 2.5 times more reactive than the $\alpha$-isomer at pH 5.5. This result is consistent with the observed $\alpha/\beta$ epoxide ratio changes which occurred in the autoxidizing cholesterol dispersions at acidic pH values. In addition, the lability of the $\beta$-isomer could account for high $\alpha/\beta$ epoxide ratios obtained from cholesterol-containing foods and heated fats which are acidic in pH. However, enzymatic and stereospecific chemical epoxidation cannot always be ruled out as alternative explanations.

## REFERENCES

1. Bergstrom, S., and O. Wintersteiner, *J. Biol. Chem. 143*:503 (1942).

2. Bergstrom, S., and O. Wintersteiner, *Ibid. 145:*309 (1942).
3. Bergstrom, S., and O. Wintersteiner, *Ibid. 145:*327 (1942).
4. Kimura, T., M. Kawata and T. Sawaya, *Chem. Pharm. Bull. 24:*2258 (1976).
5. Korahani, V., J. Bascoul and A. Crastes de Paulet, *Lipids 17:*703 (1982).
6. Norcia, L.N., *J. Amer. Oil Chem. Soc. 38:*238 (1961).
7. Muto, T., J. Tanaka, T. Miura and M. Kimura, *Chem. Pharm. Bull. 31:*1561 (1983).
8. Ansari, G.A.S., and L.L. Smith, *Photochem. Photobiol. 30:*147 (1979).
9. Kimura, M., and T. Muto, *Chem. Pharm. Bull. 28:*1836 (1980).
10. Ansari, G.A.S., and L.L. Smith, *J. Chromatog. 175:*307 (1979).
11. Smith, L.L., W.S. Matthews, J.C. Price, R.C. Bachmann and B. Reynolds, *J. Chromatog. 27:*187 (1967).
12. Gray, M.F., and T.D.V. Lawrie, *Lipids 6:*836 (1971).
13. Mosbach, E.H., M. Nierenberg and F.E. Kendall, *J. Am. Chem. Soc. 75:*2358 (1953).
14. Chicoye, E., W.D. Powrie and O. Fennema, *Lipids 3:*335 (1968).
15. Smith, L.L., in *Cholesterol Autoxidation,* Plenum Press, New York, 1981.
16. Smith, L.L., and J.C. Price, *J. Chromatog. 25:*509 (1967).
17. Peng, S.-K., and C.B. Taylor, in *World Review of Nutrition and Dietetics,* Vol. 44, edited by Bourne, G.H., and S. Karger, Basel, 1984, pp. 117–154.
18. Peng, S.-K., and C.B. Taylor, in *Dietary Fats and Health,* edited by Perkins, E.G., and W.J. Visek, American Oil Chemists' Society, Champaign, IL, 1983, pp. 919–933.
19. Taylor, C.B., S.-K. Peng, N.T. Werthessen, P. Tham and K.T. Lee, *Am. J. Clin. Nutr. 32:*40 (1979).
20. Kaunitz, H., *J. Amer. Oil Chem. Soc. 52:*293 (1975).
21. Addis, P.B., A.S. Csallany and S.E. Kindom, in *ACS Symposium Series No. 234,* Chap. 5, edited by Finley, J.W., and D.E. Schwass, American Chemical Society, Washington, DC, 1983, pp. 85–98.
22. Sevanian, A., and A.R. Peterson, *Proc. Nat. Acad. Sci. USA 81:*4198 (1984).
23. Kelsey, M.I., and R.J. Pienta, *Cancer Letters 6:*143 (1979).
24. Bischoff, F., *J. Nat. Cancer Inst. 19:*977 (1957).
25. Bischoff, F., *Adv. Lipid Res. 7:*165 (1969).
26. Black, H.S., and D.R. Douglas, *Cancer Res. 32:*2630 (1971).
27. Black, H.S., and W.-B. Lo, *Nature 234:*306 (1971).
28. Sporer, A., D.R. Brill and C.P. Schaffner, *Urology 20:*244 (1982).
29. Hill, J.C., S.-K. Peng, R.J. Morin and C.B. Taylor, *Exp. Mol. Pathol. 41:*249 (1984).
30. Baranowski, A., C.W.M. Adams, O.B. Bayliss-High and D.B. Bowyer, *Atherosclerosis 41:*255 (1982).
31. Higley, N.A., and S.L. Taylor, *Fd. Chem. Toxic 22:*983 (1984).
32. Imai, H., N.T. Werthessen, V. Subramanyam, P. LeQuesne, A.H. Soloway and M. Kanisawa, *Science 207:*651 (1980).
33. Peng, S.-K., P. Tham and C.B. Taylor, *Am. J. Clin. Nutr. 32:*1033 (1979).
34. Lee, K.T., K. Janakidevi, M. Kroms, J. Schmee and W.A. Thomas, *Exp. Mol. Pathol. 42:*71 (1985).
35. Smith, L.L., M.J. Kulig, D. Miller and G.A.S. Ansari, *J. Am. Chem. Soc. 100:*6206 (1978).
36. Gumulka, J., J. St. Pyrek and L.L. Smith, *Lipids 17:*197 (1982).
37. Smith, L.L., and M.J. Kulig, *Cancer Biochem. Biophys. 1:*79 (1975).
38. Tohma, M., T. Tomita and M. Kimura, *Tetrahedron Lett. 44:*4359 (1973).
39. Sevanian, A., J.F. Mead and R.A. Stein, *Lipids 14:*634 (1979).
40. Muto, T., J. Tanaka, T. Miura and M. Kimura, *J. Pharm. Dyn. 6:*S-15 (1983).
41. Maerker, G., and J. Unruh Jr., *J. Amer. Oil Chem. Soc. 63:*767 (1986).
42. Smith, L.L., and F. Hill, *J. Chromatog. 66:*101 (1972).
43. Reitsema, R.H., *Anal. Chem. 26:*960 (1954).
44. Wu, G.-S., R.A. Stein and J.F. Mead, *Lipids 17:*403 (1982).
45. Kimura, M., Y. Jin and T. Sawaya, *Chem. Pharm. Bull. 27:*710 (1979).
46. Smith, L.L., and M.J. Kulig, *J. Org. Chem. 38:*3639 (1973).
47. Teng, J.I., M.J. Kulig, L.L. Smith, G. Kan and J.E. van Lier, *J. Org. Chem. 38:*119 (1978).
48. Smith, L.L., J.I. Teng, M.J. Kulig and F.L. Hill, *Ibid. 38:*1763 (1978).
49. Glass, R.L., *Lipids 6:*919 (1971).
50. Fieser, L.F., and S. Rajagopalan, *J. Am. Chem. Soc. 71:*3938 (1963).
51. Davis, M., and V. Petrow, *J. Chem. Soc.* 2536 (1949).

[Received September 23, 1985]