Cholesterol Oxides III. Autoxidation of Cholesterol in Sodium Stearate and Sodium Linoleate Dispersions

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Cholesterol dispersed in water with sodium stearate at 80°C did not autoxidize in 24 hr when the cholesterol/dispersant ratio was 0.35 or below. At ratios of 0.44 and above, however, autoxidation proceeded smoothly but ceased after 60-70% of the cholesterol was oxidized. Replacement of sodium stearate as dispersant with sodium linoleate caused cholesterol to be oxidized at a cholesterol/dispersant ratio of 0.07, and gave a longer induction period and a higher conversion to 5,6-epoxide.

Aqueous dispersions of cholesterol have been, and continue to be, of interest to scientists as relatively uncomplicated model systems in which to study the oxidation of this important component of foods. The intricacies of cholesterol oxidation under a variety of conditions have been detailed (1), and the current status of cholesterol autoxidation has been summarized recently (2,3). Bergström and Wintersteiner (4-6), who conducted a systematic study of the oxidation of cholesterol in aqueous dispersion more than 40 years ago, identified the major oxidation products found, and laid the foundation of our current knowledge of the system. More recently, several investigators (7-13), using newer chromatographic techniques developed since the mid-1960's, have added to this knowledge.

Currently available information can be briefly summarized as follows:

1. The principal products resulting from air oxidation of cholesterol in aqueous media under a variety of conditions are 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), the major oxidation product (4), cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol (epimeric 7-hydroxycholesterols) (4), and cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide (epimeric 5,6-epoxides) (7). The epimeric 7-hydroperoxides are the initial oxidation products and the other products are derived from them (8). As a result, the concentration of the hydroperoxides peaks early in the reaction profile and then declines. If the oxidation is carried out in alkaline medium, some or most of the 7-ketocholesterol may dehydrate to form 3,5-cholestadien-7-one (7), while in acid media the 5,6-epoxides hydrate to form 5α-cholestane-3β,5,6β-triol (triol) (9).

2. Invariably, a significant portion of cholesterol remains unoxidized at the end of the reaction period. Attempts to drive the reaction further have failed (5,11).

3. Sodium stearate has been the dispersant of choice, but oxidation also took place when sodium cholesteryl hemisuccinate, sodium cholesteryl sulfate or various Tritons were used. On the other hand, no oxidation took place when sodium dodecyl sulfate, sodium taurocholate or several other materials were the dispersants (10).

4. Free cholesterol oxidized more rapidly than its stearate and oleate esters and at about the same rate as cholesteryl linoleate (12,13).

Most previous researchers employed dispersions in which the molar ratio of cholesterol to sodium stearate was unity (10) or greater (7,11,13,14). The current study was undertaken to study molar ratios of cholesterol to sodium stearate below unity, as well as to study the effect of replacement of a part or all of the sodium stearate with sodium linoleate.

EXPERIMENTAL

Reagents and materials. Cholesterol (99+%), linoleic acid (99%), 5α-cholestan-3β-ol-6-one (6-ketocholestanol), N,N-dimethyl-p-phenylenediamine dihydrochloride and lipoxidase (from soybean, Type I) were purchased from Sigma Chemical Co. (St. Louis, MO). Stearic acid (technical grade) was purified by double recrystallization from acetone. Triphenylphosphine (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Thin layer chromatography plates, silica gel GHL (250 μm) were purchased from Analtech, Inc. (Newark, DE). Compressed air, zero grade, was supplied by M.G. Industries (Valley Forge, PA). All solvents were distilled-in-glass grade, and all chemicals were of reagent grade quality.

Preparation of dispersions. Buffer used for making stearic acid dispersions was prepared by addition of 1 ml of a stock aqueous solution of $Na_2HPO_4.7H_2O$ (0.373 M) to 150 ml of deionized, doubly distilled water in a 250 ml Erlenmeyer flask. The buffer was heated to 80°C, and approximately 125 mg of stearic or linoleic acid, previously dissolved in 4 ml absolute ethanol, was added to the vigorously stirred solution. Sufficient aqueous sodium hydroxide solution (1N) was added to the turbid suspension to clarify it. This clear dispersion was allowed to cool to room temperature, and its pH was adjusted to 8.0 with 10% H_3PO_4. The turbid mixture was reheated to 80°C but remained turbid until the desired amount of cholesterol (dissolved with heating in 2 ml abs. ethanol) had been added with vigorous stirring, at which time it clarified. Three drops of antifoam agent (SAG-47, Union Carbide Corporation, New York, NY) were added, and a 5 ml aliquot was withdrawn immediately to serve as the unoxidized cholesterol control. The remainder of the dispersion was transferred to a four-neck, 250 ml, round bottom flask equipped with a water cooled condensor, a gas dispersion tube connected to a compressed air tank fitted with an Ascarite II trap (to remove any trace of CO_2), and a thermometer. The temperature of the reaction was maintained at 80°C ± 1°C with an I^2R Therm-O-Watch controller (Instruments for Research and Industry, Cheltenham, PA) connected to a heating mantle. The reaction apparatus was placed inside a light-proof cabinet, and aliquots (5 ml) were withdrawn periodically to monitor the progress of the cholesterol oxidation.

Isolation and measurement of oxidation products. Each aliquot sample was placed in a 24 ml screw-top vial

and was allowed to cool to room temperature. Its pH was adjusted to 9.0 with 1N NaOH, and it was blanketed with nitrogen and placed in a freezer (-85°C) for subsequent analysis. The collected aliquots were thawed and each was extracted three times with 7 ml portions of ethyl acetate. Emulsions were broken first with anhydrous sodium sulfate crystals and then by centrifuging at 200 x g for 10 min. For each aliquot the combined extracts were dried over anhydrous Na_2SO_4, transferred to another vial, and the ethyl acetate removed under a stream of nitrogen. The samples were reconstituted in 500 μl acetone, a 100 μl aliquot was taken from each, and the acetone was removed from the aliquot under nitrogen. Each residue was treated with 200 μl triphenylphosphine in benzene (25 mg/ml), and the reaction was allowed to proceed at room temperature for 1 hr. Benzene was removed with nitrogen, each residue was reconstituted with 100 μl of acetone, and 16 μl of each acetone solution was applied to a 20 x 20 cm scored silica GHL thin layer plate that had been prewashed with chloroform/methanol (2/1 v/v) and reactivated at 110°C. The TLC plates were developed with benzene:ethyl acetate:acetic acid (60:40:1, v/v/v), and one scored section was sprayed with H_2SO_4:H_2O (1:1, v/v) to locate the bands.

The area from the front of the cholesterol band to the origin was scraped, and the silica powder form each plate was placed into a vial and extracted three times with 7 ml portions of acetone. The silica gel was separated from the organic phase by centrifugation at 200 x g for 10 min. The pooled acetone extracts were evaporated under nitrogen, and the samples were reconstituted in 250 μl acetone. A portion (2 μl) of each acetone solution was analyzed by gas chromatography. Cholesterol and its oxides were quantitated as percent composition from the normalized area counts of their peaks.

Preparation of cholesterol oxides. Mixtures of cholesterol oxides were prepared by air oxidation of dispersions prepared from 125 mg stearic acid and 170 mg cholesterol in 150 ml diluted buffer at 80°C for 24 hr. The reaction mixture was allowed to cool, its pH was adjusted to 9, and the aqueous suspension was extracted three times with 250 ml portions of ethyl acetate. The pooled organic phases were dried over anhydrous Na_2SO_4 and the solvent was removed on a rotary evaporator under aspirator vacuum. The residue, a mixture of cholesterol, cholesterol oxides and stearic acid, was suspended in 5 ml ethyl acetate and the suspension added to a previously prepared column of Florisil. The latter had been prepared by washing 45 g of Florisil with 170 ml hexane, removal of the solvent by filtration and evacuation, and addition of the dry powder to a 25 x 2 cm glass column. The sample was eluted from the column with 450 ml ethyl acetate to obtain a sterol fraction essentially free of stearic acid as judged by TLC. Solvent was removed on the rotary evaporator, and the residue was solubilized with 10 ml ethyl acetate. Composition and amounts of the products were determined by on-column capillary GC with use of 6-ketocholestanol as internal standard (15).

Preparation of sodium 13-hydroperoxy-9-cis-11-trans-octadecadienoate. The method used was a modification of one described by Chan *et al.* (16). Linoleic acid (0.694 g) dissolved in ethanol (2 ml) was added to sodium borate buffer (0.1 M, 150 ml). The suspension was clarified by the addition of NaOH (0.1 N), and the pH was further adjusted to 9.0. The solution at 30°C was stirred and a slow stream of oxygen was passed through it. Soybean lipoxygenase (lipoxidase type I, 0.1271 g) dissolved in borate buffer (0.1 M, 15 ml) was added in 5 ml aliquots at 10 min intervals. After 35 min the reaction was stopped by acidification to pH 3.0 with 0.01 N HCl. The mixture was extracted with BHT-free ethyl ether (3 x 400 ml), and the combined extracts were washed with deionized water (2 x 300 ml). The solution was dried over anhydrous Na_2SO_4 and the ether removed on a rotary evaporator. The dry residue (0.732 g) was resuspended in hexane:ethyl ether (90:10 v/v) and applied to a column of silicic acid (100 mesh, 18 g) that had been dried at 110°C for three hr and had been equilibrated with hexane:ethyl ether (90:10, v/v). Elution with hexane:ethyl ether (90:10, v/v, 200 ml) to remove unreacted linoleic acid was followed by elution of the hydroperoxides with hexane:ethyl ether (70:30, v/v, 300 ml). The second fraction was freed of solvent on the rotary evaporator, and the residue, after reconstitution in acetone (3.5 ml) was analyzed by UV (16) to contain 0.315 g conjugated diene. TLC plates developed with hexane:2-propanol:acetic acid (90:10:1, v/v/v) showed the sample to contain three hydroperoxides (pink spots) on spraying with N,N-dimethyl-p-phenylene diamine dihydrochloride (17).

Gas chromatography. Gas chromatography was performed without prior silylation as descrbied previously (15).

RESULTS AND DISCUSSION

The current set of experiments was carried out at 80°C in the absence of light. Agitation was provided by a magnetic stirrer and a stream of air.

The temperature chosen for these experiments provided a convenient oxidation rate without excessive foaming. It had been previously determined (5,9) that temperature affects the reaction rate but does not alter product distribution. Others (5) have been concerned that the degree of dispersion might affect the reaction rate or its extent. The stability of the dispersion over the 24 hr reaction period was of concern here as well. We found that addition of cholesterol (dissolved in ethanol) to a preformed stearate dispersion led to a more stable dispersion than the reverse procedure. The physical state of a dispersion, i.e., its clarity, was a matter of subjective judgment. In order to introduce somewhat more objectivity into that judgment, a simple scoring system was devised. A page of standard print was reduced on a photocopier to 64, 41 and 26% of its original size, and was also enlarged to 115 and 132% of the original. To judge the clarity of a hot dispersion, 5 ml of it was pipetted into a 22 ml vial, the vial was set on the printed page, and the print was read vertically through the column of liquid. If the smallest print (26%) was readable through the liquid, a score of "6" was assigned to the dispersion, while the largest print (132%) merited only a score of "1". Most stearate dispersions were rated "6" at the beginning of the autoxidation and retained that clarity throughout the 24 hr reaction period.

A common result of previous studies has been that a portion of the cholesterol remained unoxidized, even

after 96 hr at 80°C (12). This has frequently been attributed to unspecified micellar effects (5,10,12). In the current work, sodium stearate concentrations ranged from 0.73 mM to 9.3 mM and were thus both above and below the critical micelle concentration (cmc) of sodium stearate, which has been reported to be 1.8 mM at 50°C (18). In this connection it is interesting to note that the cmc of cholesterol has been measured to be 25-40 nM at 25°C (19), a value that is exceptionally low. In the work reported here, a series of experiments was carried out at a stearate concentration of 3.0 mM, which is well above the cmc of the dispersant. Cholesterol concentrations in this set varied from 0.68 mM to 3.0 mM, so that cholesterol/stearate molar ratios at the beginning of the experiments ranged from 0.23-1.0, a region that has not been previously studied. Results are shown in Table 1. The data indicate that at cholesterol/stearate molar ratios of 0.35 or below, cholesterol is not oxidized during the 24 hr reaction period, and the physical state of the dispersions deteriorates during the same period. The lower the molar ratio, the more rapid the deterioration of the dispersion. At molar ratios of 0.44 and above, however, a significant portion (62-71%) of the cholesterol is oxidized and the dispersions are more stable.

TABLE 1

Autoxidation of Cholesterol in Aqueous Sodium Stearate Dispersions[a]

Expt no.	Cholesterol concentration mM	Cholest/stearate molar ratio	Cholesterol remaining after 24 hr		Clarity	
			Percent	mg/150 mL	Score after 24 hr	Hours to initial decrease
1	0.68	0.23	100	40	3	2
2	0.86	0.29	100	50	1	5
3	1.0	0.35	100	60	1	5
4	1.3	0.44	39	29	6	—
5	3.0	1.0	29	50	4	24
6	0.67	1.0	30	12	4	12

[a]Stearic acid concentration was 3.0 mM for Experiments 1-5, 0.67 mM for Experiment 6.

It is useful to note that previous investigators have worked with sodium stearate concentrations ranging from 0.87 mM (7) to 5 mM (10) and at cholesterol to stearate ratios from 1.0 (10) to 6.5 (14). In most cases substantial amounts of recovered, unoxidized cholesterol were noted or implied. In the current experiments unoxidized cholesterol was much in evidence, as shown in the data of Table 1. However, while the amount of stearate present in these experiments was constant (except in Experiment 6), the amount cholesterol remaining unoxidized after 24 hr was not. If one assumes that the cmc of sodium stearate at 80°C is of the same order of magnitude as that at 50°C (1.8 mM), then it would be expected that in a 3.0 mM dispersion about 60% of the stearate is in monomeric form and 40% in micellar aggregates.

If the protection of cholesterol against oxidation is attributed to micellar sodium stearate, the results of Table 1 would lead to the conclusion that a variable amount of cholesterol is protected by a constant amount of micellar sodium stearate, perhaps by altering the number and size of the micelles. The role that stearate micelles might play in preventing complete cholesterol oxidation was tested by diluting a dispersion of cholesterol in sodium stearate (Experiment 6, Table 1) so that the concentration of each component was 0.67 mM, a level at which stearate should be entirely in the monomeric form, since it is well below the cmc. After 24 hr oxidation at 80°C the amount of unoxidized cholesterol was 30% of the starting material, essentially the same as it was at higher concentration (Experiment 5, Table 1). It is concluded that micellar shielding is probably not the controlling factor preventing complete cholesterol oxidation, since the presumed presence or absence of stearate micelles did not affect the degree of oxidation. On the other hand, the fact that increasing amounts of cholesterol at cholesterol/stearate ratios of 0.35 and below seem to lend increasing stability to stearate dispersions seems to argue for the presence of the cholesterol in the stearate micelles. The incorporated cholesterol, although not oxidizable, appears to stabilize the stearate micelles; it is, in fact, known to stabilize unilamellar vesicles and other membrane-like material (20,21).

Oxidation mixtures were sampled periodically over 24 hr, and cholesterol and its principal oxidation products were determined by capillary GC without silylation (15). Results of a typical set of experiments, carried out in triplicate, are shown in Table 2.

TABLE 2

Autoxidation of Cholesterol in Aqueous Sodium Stearate Dispersions — Reaction Progress[a]

Time (hrs)	Product composition (%)			
	Cholesterol	5,6-Epoxides & triol	7ξ-Hydroxycholesterols	7-Ketocholesterol
0	100	—	—	—
2	95 ± 3	0.30 ± 0.5	3.3 ± 4	3.8 ± 5.2
4	54 ± 6	4.3 ± 1.1	22 ± 7	19 ± 5
6	35 ± 1	7.1 ± 1.6	20 ± 4	38 ± 6
8	28 ± 2	7.0 ± 0.7	19 ± 1	47 ± 0.5
12	31 ± 0.5	5.9 ± 0.8	16 ± 1	47 ± 0.7
24	27 ± 3	4.7 ± 1.4	18 ± 1	46 ± 3

[a]Cholesterol and sodium stearate:3.0 mM each. n=3.

TABLE 3

Autoxidation of Cholesterol in Aqueous Sodium Linoleate Dispersions[a,b]

Expt no.	Cholesterol concentration mM	Cholest/linoleate molar ratio	Cholesterol remaining after 24 hr		Clarity score after 24 hr
			Percent	mg	
1	0.72	0.07	25	11	5
2	0.69	0.23	59	24	4
3	1.3	0.44	42	32	3
4	3.0	1.0	51	87	5

[a]Reaction: 24 hr at 80°C.
[b]Linoleic acid concentration: 10.1 mM for Experiment 1, 3.0 mM for Experiments 2–4.

Oxidation reached a maximum after 8-12 hr, but the reaction was continued to 24 hr to assure maximum cholesterol conversion. 7-Ketocholesterol was the principal oxidation product, as noted by others (4), and comprised about one-half of the total oxidation products. The epimeric 7-hydroxycholesterols appear to increase first and then to decrease. However, the apparent peaking of these compounds is an artifact of the methodology used which included treatment of the samples with triphenylphosphine to reduce the epimeric 7-hydroperoxycholesterols to the 7-hydroxy derivatives. The hydroperoxides are the initial oxidation products of cholesterol. They peak early in the oxidative process and have disappeared completely after 24 hr (9). The rise and fall in hydroperoxide concentration is superimposed on the rising 7-hydroxycholesterol concentration, and the sum of the two is reflected in the data shown in Column 4 of Table 2.

Most of the aliquots sampled from the reaction mixture 8 hr or more after the beginning of the oxidation contained small amounts of triol. The latter is a hydration product of either of the isomeric 5,6-epoxides, and hence the sum of the epoxides and the triol are presented in a single column in Table 2.

The cholesterol used in these experiments was purchased as a 99±% grade and was not purified further. Analyzed by our procedure, it contained no detectable amounts of the cholesterol oxidation products of interest. Other impurities present in the starting material may have been responsible for the considerable variation in the induction period and in the rate of oxidation in the early hours of replicate experiments (Table 2). Similar variations have been encountered by other investigators (11,12). Poor reproducibility in product compositions in the early hours of the oxidation reaction were also encountered in this laboratory at lower cholesterol/stearate ratios (data not shown).

As expected, an increase in the flow of air through the reaction mixture from 200 ml/min to 400 ml/min had no noticeable effect on product composition or on the percent of cholesterol oxidized. Kimura (11) had shown earlier that air flow through the reaction mixture was not required, and that oxidation proceeds without further addition of oxygen, attaining about the same endpoint observed here. In the current set of experiments, airflow was used not only to replenish the oxygen supply in the dispersion, but also to provide additional agitation.

Replacement of sodium stearate with sodium linoleate as dispersant caused several changes in the reaction of the dispersed cholesterol. Most noticeable was an unexpected increase in the induction period of cholesterol oxidation. After three hr at 80°C all of the cholesterol remained unoxidized, and none of its oxidation products were detectable in the mixture. The reason for the increased induction period is not clear. Linoleate oxidizes more rapidly than oleate (23,24) and also more rapidly than cholesterol, which, like oleate, contains a single double bond. Hydroperoxy radicals derived from linoleate might act as initiators for cholesterol autoxidation. This would result in a shorter induction period for cholesterol in linoleate than in stearate dispersion. The opposite was observed. Apparently there is preferential oxidation of linoleate while the oxidation of cholesterol is delayed.

TABLE 4

Autoxidation of Cholesterol in Aqueous Linoleate/Stearate Dispersions

Expt no.	Dispersant[a] Linoleate:stearate %	Cholesterol	Product composition (%)[b]			Ratio 7-keto: epoxides & triol
			5,6-Epoxides plus triol	7-Hydroxycholesterols	7-Ketocholesterol	
1	100:0	42	9.7	14.8	32.6	3.4
2	50:50	59	4.8	10.7	24.4	5.1
3	25:75	64	4.2	6.2	25.3	6.0
4	0:100	39	3.7	15.6	41.2	11.1
5	100:0	25	16.4	11.9	47.9	2.9

[a]Cholesterol/dispersant ratio: 0.44 for Experiments 1–4; 0.07 for Experiment 5
[b]Reaction: 24 hr at 80°C.

Regardless of the extended induction period, however, the percentage of unoxidized cholesterol remaining after 24 hr was about the same for stearate or linoleate dispersions when the molar ratio of cholesterol:fatty acid was the same. A comparison of Tables 1 and 3 shows this more clearly.

Although the amount of buffer present was the same in stearate and linoleate dispersions, the pH of the sodium linoleate dispersion of cholesterol decreased as the oxidation reaction progressed. It reached a final value of 6.6 after 24 hr at 80°C in 3.0 mM sodium linoleate. Equivalent sodium stearate dispersions maintained the starting pH throughout the 24 hr period. The pH decrease in the dispersion of the polyunsaturated fatty acid presumably is caused by the accumulation of short chain alkanoic acids (25) from the decomposition of fatty acid hydroperoxides under oxidizing conditions. The pH of sodium linoleate alone (no cholesterol) under the same conditions decreased to 5.2 in 24 hr.

When sodium linoleate was the dispersant in place of sodium stearate the epoxide content of the oxidation products increased and 7-ketocholesterol content decreased (Table 4). The increase in epoxide content is interesting, since it implicates involvement of linoleate hydroperoxide or a derived radical in the cholesterol oxidation process and in direct attack on the cholesterol double bond. Attack on the double bond as well as on the allylic C-7 position of cholesterol by cholesterol hydroperoxides (26), long chain fatty acid hydroperoxides (27) and various alkyl and arylhydroperoxides (28) has been reported to occur in aprotic solvents. In water, however, attack on cholesterol by hydroperoxy radicals derived from linoleate apparently does not occur because of the ready availability of hydrogen radicals from the solvent, unless there exists within the aqueous system a hydrophobic microenvironment. Within such a lipophilic domain, fatty acid hydroperoxy radicals and cholesterol might coexist in close enough proximity to react. The increase in cholesterol epoxides content in linoleate dispersions compared to that of stearate dispersions is consistent with the existence of such a microenvironment. Support for this concept also comes from an experiment in which the cholesterol to linoleate molar ratio was substantially decreased (Experiment 5, Table 4). In this experiment, 75% of the cholesterol oxidized in the 24 hr period, but more importantly, the epoxide plus triol content of the oxidation products reached 15%. When linoleate was replaced with stearate under otherwise identical conditions (molar ratio 0.07), cholesterol was not oxidized.

Linoleate hydroperoxides, once formed, do not appear to oxidize cholesterol in aqueous dispersions. No oxidation of cholesterol occurred when sodium 13-hydroperoxy-9-*cis*-11-trans-octadecadienoate that had been prepared separately was used as dispersant either alone or with equal amounts of sodium stearate. These experiments were carried out under nitrogen and at 80°C for 12 hr. At the end of this period no hydroperoxides were left in the dispersions as determined by spraying developed TLC plates of the reaction mixtures with N,N-dimethyl-p-phenylene diamine hydrochloride solution. It is concluded from the present work that microenvironment plays a significant role in the oxidation of cholesterol in aqueous dispersions, and hence may be important in the oxidation of sterols in food systems. Failure of cholesterol to oxidize in stearate dispersions when the molar ratio of cholesterol/dispersant is below 0.4, while in linoleate dispersion this limit does not seem to exist, raises interesting questions regarding the structure of the micelles in these dispersions. Further work is required to resolve these questions.

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