Concurrent Oxidation of Cholesterol and Corn Oil Sterols in Autoxidizing Lipid Films[1,2]

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A new, simple method based on assay of cholesterol is presented for study of the time course and extent of air oxidation of cholesterol. Using such a method, the air oxidation of 0.5–3.5% solutions of corn oil sterols and cholesterol in films of either corn oil or corn oil fatty acids has been followed by the author for periods of up to 3 months at room temperature and 2 weeks at 60°C. in the dark. Under these conditions it was shown that a) solid cholesterol is relatively stable to air oxidation; b) in lipid films cholesterol is readily subject to oxidative attack which appears to be closely linked to the stability of the lipid film itself to autoxidation; c) more than 90% conversion of cholesterol to oxidation products has been observed in such systems; d) the principal corn oil sterols are oxidized at the same rate as cholesterol, and esterification of the corn oil sterols did not affect the rate at which they were oxidized; and e) the rate of concurrent oxidation of cholesterol in corn oil and corn oil fatty acid films is independent of the concentration of cholesterol at low concentrations, 0.5–3.5%. The disappearance rate is dependent on the stability of the lipid film itself as shown by peroxide value.

IN A PREVIOUS STUDY (1) it was shown that aeration of cholesterol in an aqueous colloidal suspension stabilized with sodium stearate resulted in percentage losses in assay of cholesterol by the Sperry-Webb (2) modification of the Schoenheimer-Sperry method (3), which were approximately equal in amount to the percentage of conversion of cholesterol to diols (7-hydroxy-cholesterol epimers) and ketone (7-ketocholesterol). 7-Ketocholesterol is precipitable with digitonin under some conditions (4) but is inactive to the Liebermann-Burchard reagent (5). 7-Hydroxycholesterol, on the other hand, produces about three times as much color as cholesterol (6,7) under conditions similar to those of the Sperry-Webb method but has been shown (7) not to be precipitable with digitonin under the conditions of precipitation used in the Sperry-Webb method. The minor product of air oxidation of cholesterol, $3\beta,5\alpha,6\beta$-cholestanetriol, yields no color with the Liebermann-Burchard reagent (8). All of these findings together suggest that the Sperry-Webb method for assay of cholesterol should be valuable as a new, simple tool to follow the time course and extent of air oxidation of cholesterol, at least under conditions in which compounds with oxygen functions at carbons 5, 6, and 7 are the principal products.

By using the Sperry-Webb assay procedure for cholesterol, the air oxidation of 0.5–3.5% solutions of corn oil sterols and cholesterol in films of either corn oil or corn oil fatty acids has been followed by the author for periods of up to three months at room temperature and two weeks at 60°C. in the dark. In this report it is again demonstrated that solid cholesterol is relatively stable to air oxidation but that, when dissolved in films of unsaturated fatty acids (obtained by saponification of corn oil, a process involving concomitant elimination of most of the antioxidants present in the intact oil), cholesterol is readily air-oxidized at room temperature. While cholesterol in films of corn oil itself is not air-oxidized at room temperature, oxidation proceeds rapidly at 60°C.

Materials and Methods

Concurrent Oxidation at Room Temperature. Air oxidation of U.S.P. cholesterol in Mazola[4] brand corn oil, and in fatty acids derived therefrom, was followed for three months at 21–25°C. by measuring cholesterol losses with the Sperry-Webb (2) modification of the Schoenheimer-Sperry method (3). The corn oil fatty acids were prepared by saponifying corn oil in ethanol with KOH by a quick saponification procedure (5 min. of boiling). After cooling, the soaps were extracted with petroleum ether to remove unsaponifiables and acidified with HCl; the free fatty acids were taken up in petroleum ether and washed with water until the washes were neutral to Methyl Orange; the solvent was finally removed under reduced pressure. All of the above processes and operations were carried out under a blanket of oxygen-free nitrogen. The fatty acids so obtained still contained some unsaponifiables, shown by residual sterol assayed as cholesterol. Nonetheless the alkali treatment had apparently destroyed most of the antioxidants since the fatty acids were found to be much less stable to autoxidation than the parent oil.

For the oxidation studies, series of triplicate 25-ml. volumetric flasks were used to which separate aliquots of cholesterol and corn oil, or fatty acids in organic solvent were added. The solvent was removed with a stream of oxygen-free nitrogen. The first flask of a triplicate contained 2.5 mg. of solid cholesterol, the second 2.5 mg. of cholesterol in 100 mg. of corn oil or fatty acids, and the third 100 mg. of corn oil or fatty acids. All flasks were carefully wrapped in aluminum foil to exclude light, stoppered loosely with a plug of absorbent cotton, and stored in a dark cabinet drawer at room temperature. At different time-intervals triplicate groups were assayed for cholesterol. Assay of sterol calculated as cholesterol in the third flask was used to correct the cholesterol value of the second flask for plant sterols assayed as cholesterol.

Peroxide accumulation was studied in separate 5-g. samples of corn oil or fatty acids in 125-ml. Erlenmeyer flasks stored under the same conditions as the volumetric flasks. Peroxide value was determined by an iodometric method (9).

Concurrent Oxidation at 60°C. Studies similar to the room temperature experiments were carried out

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at elevated temperature, using a forced draft oven thermostatically controlled at 60°C.

Results

Data from the room temperature experiments are presented in Table I. These data show that the cholesterol present in corn oil was not oxidized insofar as shown by assay but that about 92% of the cholesterol originally present in the corn oil fatty acids was converted to oxidation products. The peroxide values indicate the relatively greater instability of the fatty acids compared with the oil. The peroxide values shown in this table cannot be correlated directly to the assay losses of cholesterol since the peroxide values were determined on 5-g. samples of lipid. At 60°C. peroxide accumulation was shown to be much more rapid for 100-mg. samples of lipid in 25-ml. volumetric flasks than for 5-g. samples of lipid in 125-ml. Erlenmeyer flasks. A similar comparison at room temperature was not made; thus, at any given time interval, the peroxide concentration for the sterol-assay flask was probably greater or equal to that for the P.V. flask (5 g., 125 ml.). The data of the experiments show further that the oxidation of cholesterol in such mixtures is linked to the stability of the companion lipid and that cholesterol itself stored alone under these conditions is relatively stable.

Data from the studies at 60°C. are presented in Table II. The relative stability of solid cholesterol is again shown by lack of assay losses for the 13-day interval. In corn oil about 94% conversion of cholesterol to oxidation products had occurred in 10 days, and in corn oil fatty acids about 96% conversion in eight days.

TABLE I

Concurrent Oxidation of Cholesterol Dissolved in 100 mg. of Corn Oil (C.O.) and 100 mg. of Corn Oil Fatty Acids (C.O.F.A.) at Room Temperature (21–25°C.)

Time of storage	Cholesterol alone	Cholesterol in C.O.F.A.	P.V.[a] of C.O.F.A.	Cholesterol in C.O.	P.V.[a] of C.O.
	mg./flask	*mg./flask*		*mg./flask*	
Zero	2.36	2.26	<1	2.36	<1
2 days	.....	2.27	.....	2.25	....
1 week	.....	2.30	.....	2.27	....
2 weeks	.....	2.14	8.5	2.33	2.0
1 month	2.53	1.06	23.2	2.31	2.0
2 months	.....	0.52	382.0	2.47	3.3
3 months	2.40	0.19	365.0	2.35	7.2

[a] Peroxide value in milli-equivalents of peroxide per 1,000 g. of sample.

TABLE II

Concurrent Oxidation of Cholesterol Dissolved in 100 mg. of Corn Oil (C.O.) and 100 mg. of Corn Oil Fatty Acids (C.O.F.A.) at 60°C.

Time of storage	Cholesterol alone	Cholesterol in C.O.F.A.	Cholesterol in C.O.	P.V.[a] of C.O.
	mg./flask	*mg./flask*	*mg./flask*	
Zero	2.81	2.69	2.70	<1
1 day	2.47	1.71	2.39	86
2 days	2.68	0.84	1.54	1,533
3 days	2.71	0.79	1.16	1,457
4 days	2.59	.....	0.64	1,088
5 days	.....	0.65	.....	.....
6 days	2.56	0.28	0.65	386
8 days	2.87	0.10	0.27	292
10 days	2.71	.....	0.17	338
13 days	2.75	.....	.....	.....

[a] Peroxide value in milli-equivalents of peroxide per 1,000 g. of sample.

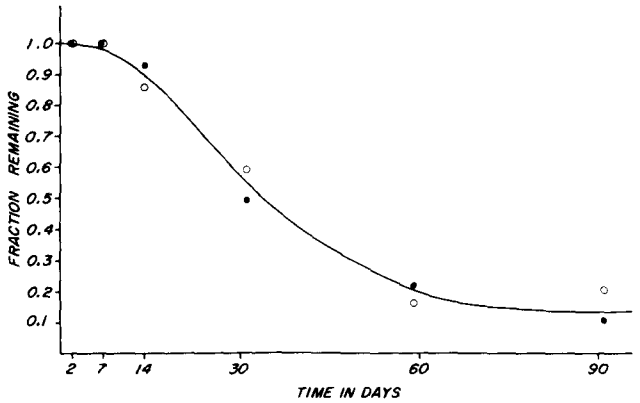

FIG. 1. Fraction of cholesterol and phytosterols remaining in autoxidizing corn oil fatty acids at room temperature: ●, cholesterol plus phytosterols, initial concentration in fatty acids 2.8% (W/W); ○, phytosterols, initial concentration in fatty acids 0.5% (W/W).

The peroxide values shown for corn oil in this table were obtained from 100-mg. samples of corn oil in 25-ml. volumetric flasks stored under the experimental conditions and hence can be directly correlated with the assay losses for the corn oil series. These data show that oxidation of cholesterol begins before the P.V. reaches 100 and continues well beyond the point of secondary decomposition of peroxides. This is evidence that cholesterol in such a system is oxidized by propagative reactions. Peroxide values on separate 5-g. samples of corn oil and fatty acids showed the fatty acids to be much less stable than the oil under these conditions. This is reflected in the greater rate of oxidation of cholesterol in the corn oil fatty acid system and is further evidence of the propagative nature of the reactions oxidizing cholesterol in such a system.

The disappearance of sterols from the systems studied is shown graphically as a function of time in Figures 1 and 2. The total sterol, cholesterol plus phytosterol values, are plotted instead of cholesterol values since this minimizes errors of chemical analysis. Total sterols[5] were determined by assay of Flask 2 of a triplicate, phytosterols by assay of Flask 3, and cholesterol values were obtained by difference. This method of treating the data is further justified by the fact that, in the study being reported, it was shown that the rates of disappearance of cholesterol and corn oil phytosterols were the same and were not affected by esterification of the sterols.

In these figures the time course of disappearance for the corn oil sterols (Flask 3), present in a 4–5 fold lesser concentration than the total sterols (Flask 2), is shown to be the same as that for the total sterols, indicating an apparent independence of the reaction on concentration of sterol for this range of concentration and under these conditions. The dif-

[5] To the author's knowledge no studies have been made and reported where the Sperry-Webb method for cholesterol has been applied to the assay of sterols in corn oil. Using this method, the author has been able to show a content of 0.80–0.92% sterols calculated as cholesterol in refined corn oil. This may be compared with a content of 0.92–1.06% sitosterols in refined corn oil reported by Rathmann (10). It is known (11) that oxidation of the C-17 side chains of cholesterol stigmasterol, and the sitosterols to the 17-keto derivative yields dehydroepiandrosterone in each case except the α-sitosterols, which appear to have a second double bond in the tetracyclic nucleus. Recently however Avigan *et al.* (12) have reported somewhat different spectral absorption of 635 and 420 mμ for cholesterol and desmosterol (24-dehydrocholesterol) after color development with the Liebermann-Burchard reagent, indicating that C-17 side chain unsaturation may affect chromogenicity.

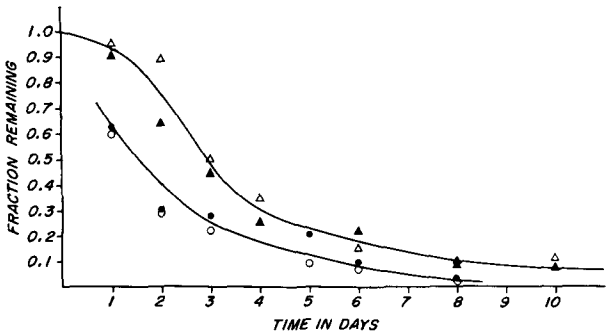

FIG. 2. Fraction of cholesterol and phytosterols remaining in autoxidizing corn oil and corn oil fatty acids at 60°C. *Corn oil study*, ▲, cholesterol plus phytosterols, initial concentration in corn oil 3.5% (W/W); △, phytosterols, initial concentration in corn oil 0.8% (W/W). *Corn oil fatty acids study*, ●, cholesterol plus phytosterols, initial concentration in fatty acids 3.3% (W/W); ○, phytosterols, initial concentration in fatty acids 0.6% (W/W).

ference in rate of disappearance of sterols from corn oil and corn oil fatty acids shown in Figure 2 indicates the dependence of the rate on the stability of the lipid film itself. The same rate of disappearance of cholesterol and phytosterols is also shown indirectly in these curves.

It may be observed in Figure 2 that the phytosterols disappear at the same rate as total sterols (mostly free cholesterol) from both the corn oil and corn oil fatty acid systems. In the fatty acids the phytosterols were present as free sterols while in the corn oil the ratio of ester phytosterol to free phytosterol was found to be 2.1 by the Sperry-Webb (2) method. These data indicate that the rate of oxidation of these sterols in such systems is not affected by esterification. This is quite different from oxidation of such sterols in aqueous colloidal suspensions since esterification has been found (13) greatly to diminish susceptibility to attack by oxygen in such a system.

Discussion

During assay of cholesterol by difference in the autoxidized mixtures by the Sperry-Webb method separate aliquots of the solutions of the lipids were also analyzed for cholesterol by difference, using the Liebermann-Burchard reaction alone (Sperry-Webb conditions) without going through the digitonide. Analysis of the mixtures by difference, using the Liebermann-Burchard reaction alone, would correct the values obtained for color produced by the fatty acid moieties and would yield some clue as to the chromogenicity of the steroid air-oxidation products obtained under these conditions. Higher values for "cholesterol" for the number two flasks were invariably obtained in this way (suggesting the presence of 7-hydroxycholesterol), but in no case did the value for any of the autoxidized mixtures exceed that for the unoxidized zero-time sample. Also fast-reacting characteristics to the Liebermann-Burchard reagent [cf. Baumann *et al.* (5,6)] were observed with the autoxidized mixtures, suggesting the presence of 7-hydroxycholesterol as an oxidation product.

The findings reported here that solid cholesterol appears to be relatively stable to air oxidation are in agreement with the findings of Dauben and Payot (14) for crystalline cholesterol that is not radioactively labelled. Susceptibility of cholesterol to oxidative attack during concurrent oxidations suggests that care must be exercised in defining the susceptibility of pure cholesterol to air oxidation as compared with that of a sample of cholesterol contaminated with unsaturated lipid.

Studies of the air oxidation of cholesterol and its esters in a variety of physical systems and chemical environments are being continued by the author. More knowledge of the reactivity of cholesterol to oxygen and free-radical attack in systems simulating in part the physical and chemical state of cholesterol and its esters in tissues may contribute information basic to the metabolism of cholesterol in both normal and disease conditions.

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