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The Influence of Autoxidation on the Chemical Assay of Cholesterol[1]

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MANY WORKERS specify specially purified cholesterol for use as a standard for analytical procedures (1–6). This stems from the known instability of cholesterol under some conditions of exposure to light and air (1, 2, 7, 8a), the crystallization of cholesterol as a hydrate from some solvents (8b, 9), and the presence of steroid impurities in commercially available cholesterol (8c, 10–14). Since most analytical procedures for cholesterol employ the relatively nonspecific Liebermann-Burchard reaction, it was desired to ascertain what influence such factors as long storage, autoxidation, and steroid impurities might have on the suitability of a sample of cholesterol for use as a standard for assay purposes.

Recently Sperry and Brand (15) reported losses of up to 25% of cholesterol when assaying "dried" serum lipides which had been exposed to air. The losses were attributed by these authors to oxidative degradation although the partial insolubility of the lipides also reported occurring under those conditions may have been causative. It was one of the purposes of this study to autoxidize cholesterol directly by a proven procedure and then to observe the effects on the chemical assay of cholesterol by standard methods.

Cholesterol autoxidation and factors governing it have been studied in detail by Bergström and Wintersteiner (16–20). Since the reaction mechanism proposed by these authors involving intermediary formation of a hydroperoxide group on a methylenic carbon atom α to the double bond is similar to the mechanism proposed by others (21) for autoxidation of methyl oleate, it was desired to test whether or not linoleate would propagate the autoxidation of cholesterol as has been noted (22) to be the case for oleate. This consequence would be of importance in assay work since cholesterol occurs in biological materials accompanied by polyunsaturated fatty acids, either as the ester or as companion mixed lipides. The suggestion of Fieser (13) that an unsaturated companion-sterol of cholesterol might be autoxidized by action of lard peroxides on it lends support to this possibility. As a part of the study being reported here, the influence of linoleate on the autoxidation of cholesterol in organic solvent, in lipide films, and in the colloidal system described by Bergström and Wintersteiner is being studied in this laboratory.

Materials and Methods

The cholesterol samples used are described in Table I.

TABLE I

Cholesterol Samples [a] Used

Designation	C-1	C-2	C-3
Description	Merck, U.S.P.[b]	Eastman Kodak Co. Cat. No. 909	Purified cholesterol[c]
Melting point, °C.	145.5–146.5	144.5–146	146–147
Specific rotation in chloroform $[\alpha]_D^t$ t	−42°, −41°	−41°, −43°	−38°, −39°
Specific rotation in chloroform $[\alpha]_D^t$ C in %	29, 30.5	28, 27.5	31.5, 32.5
	1.06, 1.04	1.15, 1.01	1.10, 1.15
Analysis—$C_{27}H_{46}O$			C 84.01, H 11.93
Calculated: C 83.86, H 11.99	—	—	C 83.93, H 12.02

[a] All melting points are uncorrected. Microanalyses and optical rotations are by E. W. D. Huffman, Denver, Colo.
[b] This sample had been stored in an amber bottle at room temperature in the laboratory for more than two years.
[c] Cholesterol (C-2) was brominated by the Windaus (23) procedure as modified by Schwenk and Werthessen (24). The dibromide was recrystallized twice, m.p. 115°C., from ethylene dichloride and then debrominated by the method of Fieser as described by Schwenk and Werthessen (24). The recovered cholesterol was recrystallized three times from absolute methanol without change in m.p., 146–147°C.

Recovery of Cholesterol Following Refluxing with Organic Solvent in the Presence of a Readily Autoxidizable Lipide Substrate. Two standard solutions in ethanol-ether (3:1 v/v) were prepared, one of corn oil (Mazola) and the other of cholesterol (C-1). Suitable aliquots of the solutions were placed in separate flasks so as to permit refluxing of the cholesterol solution, cholesterol solution plus corn oil solution, and corn oil solution. Following a reflux period of 4 hrs. under Allihn condensers open to the atmosphere, the flask contents were analyzed in duplicate for total cholesterol by the Sperry and Webb (1) modification of the Schoenheimer and Sperry (3) method and compared with a similar analysis of an unrefluxed control of the standard cholesterol solution.

Autoxidation of Cholesterol in Aqueous Colloidal Suspension. Cholesterol (C-1) in aqueous colloidal suspension stabilized with sodium stearate was autoxidized for 3½–4 hrs. by aeration according to the "standard conditions" described by Bergström and Wintersteiner (19) at pH 8.2. Two 25-ml. aliquots of mother liquor were removed at zero time and again, following the 3½- to 4-hr. aeration period. These aliquots were acidified and analyzed for total lipide, diols,[2] and ketone by methods described by Bergström and Wintersteiner (19). The lipide extracts of the aliquots were analyzed for cholesterol (both free and total) according to the Sperry and Webb modification (1) of the Schoenheimer-Sperry (3) method, also by simply applying the Liebermann-Burchard color reaction with the reagent and conditions of Sperry and Webb (1) and alternatively of Abell *et al.* (5) for color development. Infrared

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[2] The color produced by the chromogenic diols, using the Lifschütz reaction, was measured at 620 mμ. The S-62 filter used by Bergström and Wintersteiner in the Zeiss step-photometer had a maximal transmission at 619 mμ (25).

spectra of the recovered lipides were obtained by using the potassium bromide disk technique (0.1% in KBr, 2–15 μ).

Results

The purified cholesterol (C-3) was compared with the two commercial preparations by infrared study (2 μ– 15 μ) in carbon disulfide and by the potassium bromide disk technique. The spectra of the three preparations showed no differences. The purified cholesterol had a higher melting point and lower specific rotation than the other samples. The three cholesterol samples were then compared as standards for the Schoenheimer-Sperry procedure for assay of cholesterol. Absorbancy indices [3] for all three samples were identical and yielded the specific extinction-coefficient[4,5] (625 mμ) k of 4.35, which is identical to that calculated from the data of Schoenheimer and Sperry (3). The absorbancies shown by the cholesterol standard tubes of 25 randomly selected consecutive routine analytical runs in this laboratory were used to calculate k. The mean value for k and the standard error of the mean was 4.344 ± 0.022.

The values obtained from the experiment in which a solution containing cholesterol and corn oil was refluxed are given in Table II.

TABLE II

Recovery of Cholesterol Following Refluxing with Ethanol-Ether in the Presence of Corn Oil.

Sample No.	Description	Materials aliquoted into flask			Cholesterol recovered
		Solvent	Corn oil	Cholesterol	
		ml.	*mg.*	*mg.*	*mg.*
R_1	Cholesterol	20	None	1.047	1.046
R_2	Cholesterol plus corn oil	20	11.6	1.047	1.112[1]
R_3	Corn oil	20	11.6	None	0.069[1]
R_4	Cholesterol control (not refluxed)	—	None	1.047	1.060

[1] Digitonin-precipitable, Liebermann-Burchard-positive material calculated as cholesterol.

The data of Table II show that there is no loss of cholesterol as assayed by the Schoenheimer-Sperry procedure, but they do not preclude the possibility that autoxidation of cholesterol may have occurred under these conditions.

The results obtained from the experiment on the autoxidation of cholesterol in aqueous colloidal suspension are given in Table III.

TABLE III

Analysis of Mother Liquor Before and After Aeration

Measured value	Aliquots taken at zero time		Aliquots taken following 3½–4 hrs. aeration	
	a	*b*	*c*	*d*
Total lipide (mg.)	27.3	27.1	17.2	17.3
Total sterol[a] (mg.)	22.3	22.1	14.0	14.1
Diol (mg.)	None	None	2.85	2.85
Ketone (mg.)	0.17[b]	0.14[b]	6.13	5.60
Cholesterol (mg.) Schoenheimer-Sperry procedure	22.6	24.1	4.6	5.1
"Cholesterol" (mg.) Liebermann-Burchard reaction[c]	23.2	23.5	13.7	14.2
"Cholesterol" (mg.) Liebermann-Burchard reaction[d]	23.6	23.6	—	14.4

[a] Sterol calculated as cholesterol from the known proportions of cholesterol and sodium stearate added to the reaction flask and from the weight of total lipide obtained gravimetrically.
[b] This is probably *not* ketone and very likely represents nonspecific background absorption (see text).
[c] Sperry and Webb (1) conditions.
[d] Abell *et al.* (5) conditions.

[3] Absorbancy and extinction coefficient as used in this paper will refer to cholesterol, following color development according to the conditions of Sperry and Webb (1).
[4] Specific extinction-coefficient $k = (-\log_{10} T \text{ soln.}/T \text{ solv.})/bc$, b in centimeters, and c in grams cholesterol per liter.
[5] Wavelength calibration and photometric scale of the spectrophotometer used were checked by standard procedures.

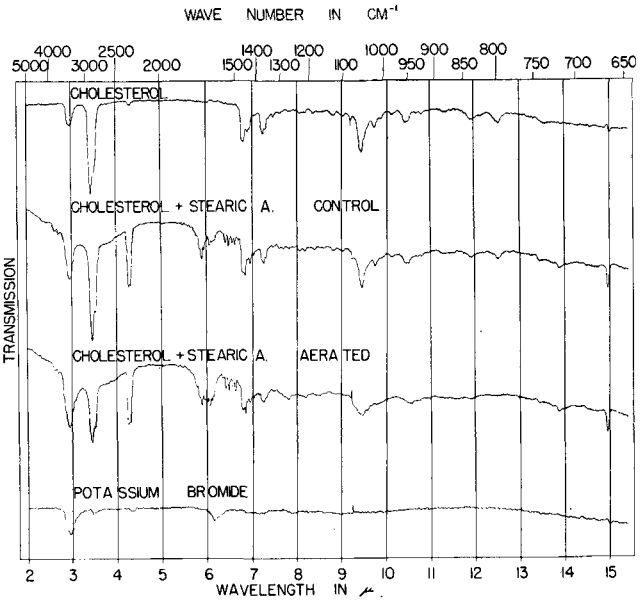

FIG. 1. Infrared spectra. Potassium bromide disk technique, 0.1% lipide in KBr.

The values given in Table III show less lipide to be present in the aliquots taken following aeration than in those taken at zero time. This is caused by the deposition of cholesterol and stearate on the walls of the reaction flask and on the stirring rod. The amounts of stearic acid in the several aliquots were not determined, hence these data do not indicate whether the relative proportions of stearate and sterol in the mother liquor are changed following aeration. Assuming no change in the proportion of sterol to stearate in the mother liquor [*cf.* Bergström and Wintersteiner (19)] following aeration, the data show an average of 20.4% conversion to diol and 41.8% conversion to ketone following aeration. This is in agreement with average values of 20 and 40%, respectively, reported by Bergström and Wintersteiner (19). The small amount of ketone tabulated for zero time indicates a small amount of absorption at 240 mμ shown by the material obtained from the aliquots taken at zero time. This is probably nonspecific background absorption because of stearic acid and cholesterol since absorption measurements were made against an alcohol blank. No absorption measurements were made at wavelengths above and below 240 mμ to check for nonspecific absorption.

The results show a loss of cholesterol assayed by the Schoenheimer-Sperry procedure (values for free cholesterol and total cholesterol were the same), which is roughly equal to the amount of cholesterol converted to ketone and diol. There is no apparent loss of cholesterol assayed by the Liebermann-Burchard reaction. This observation is explainable by the proportions of diol and ketone present and by the findings of Baumann and associates (26, 27) that 7-ketocholesterol is inactive so far as the Liebermann-Burchard reagent is concerned while 7-hydroxycholesterol gives three times as much color as cholesterol under conditions similar to those used in the work reported here.

The infrared spectra of the purified cholesterol (C-3) and of the cholesterol plus stearic acid obtained before and after aeration are given in Figure 1. The following interpretation of the infrared bands is

based in part on the literature references cited. Autoxidation resulted in diminution of absorption at 11.9 and 12.6 μ (28) because of the cholesterol double bond. Changes are also shown in the bands at 9.5–10.5 μ in the "finger-print region" characteristic of hydroxysteroids (28). Increased absorption at 6.1 μ is shown, following aeration. This likely results from carbonyl absorption of 7-ketocholesterol (29). The character of the bands at 3.0 and 3.5 μ resemble those shown for 4-hydroxycholesterol (29) more than those for cholesterol.

Bands peculiar to stearic acid are shown at 5.9 μ [carboxyl (30, 31)], 6.4–6.8 μ [stretching vibrations of CH_2 deformations (30, 31)], and 13.9 μ [methylene wagging (30, 32)]. The reason for the band at 4.3 μ shown for cholesterol plus stearic acid before and after aeration has been found to be caused by the adjustment of the infrared spectrophotometer.

Discussion

The agreement in the specific extinction coefficients of purified cholesterol and of two commercially available cholesterol preparations and the identity of the coefficient obtained with that calculated from the data of Schoenheimer and Sperry (3) suggest that the specific extinction-coefficient thus obtained is an adequate criterion for determining the suitability of a sample of cholesterol for use as a standard for analytical procedures. If a sample of cholesterol is found to yield a specific extinction-coefficient agreeing within 1 or 2% of $k = 4.35$, it should be adequate for use as a standard for assay of cholesterol in biological materials. There are several advantages in using the specific extinction-coefficient in lieu of a rigorous purification to insure the adequacy of a sample of cholesterol as a standard. It may be used as a standard without prior purification as long as it continues to yield the same absorbancy value. Sperry and Webb (1) recommend the purification of the cholesterol sample prior to preparation of the stock standard solution. This is often too cumbersome for most work; and, in addition, putting a compound through a purification procedure does not necessarily guarantee purification. Purification should be followed by the determination of physical and chemical constants on the product. Assuming that a highly purified cholesterol sample is available, the specific extinction-coefficient must then be relied upon to test the adequacy of the reagents (a critical consideration) for proper color development. In these times when good instrumentation is commonplace in most laboratories it appears that determination of the specific extinction-coefficient is the most simple and direct approach to determining the adequacy of a cholesterol sample for use as a standard for assay of cholesterol in biological materials.

There were no losses of cholesterol assayed by the Schoenheimer-Sperry method when refluxed in ethanol-ether with corn oil. The finding that autoxidation of cholesterol in aqueous colloidal suspension does result in losses of cholesterol when measured in this manner supports the contention that cholesterol was not autoxidized by refluxing in the presence of corn oil.

Summary

Cholesterol purified *via* the dibromide has been compared with two commercial preparations for use as standard materials for assay of cholesterol in biological materials. It is concluded that determination of the specific extinction coefficient, following application of the Liebermann-Burchard reaction under specified conditions, is sufficient to ascertain adequacy of a sample of cholesterol for use as a standard.

Autoxidation of cholesterol by aeration in colloidal aqueous suspension resulted in losses of assay of cholesterol by the Schoenheimer-Sperry procedure which were roughly equal in amount to the percentage of conversion of cholesterol to diols and ketone. Assay of the same material by the Liebermann-Burchard reaction alone resulted in no apparent losses through autoxidation. Cholesterol was not autoxidized by refluxing in ethanol-ether in the presence of corn oil.

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