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Effect of Lyophilization and Chloroform Extraction on Electrophoretic Migration of Serum Lipoproteins.* (22328)

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It has been shown that when serum from normal human subjects was lyophilized and extracted with cold chloroform for 3 hours only a small percentage of the total serum cholesterol was extracted(1). This fraction has been designated the "readily extractable cholesterol"—REC. The value for the REC fraction was shown to be 40 mg % or less in 98% of 687 subjects between the ages of 17-33 years, but the percentage of subjects with extraction values above 40 mg % was found to increase up to about the fifth decade(2,3). This was especially marked in the executive and professional groups studied. Subjects with coronary infarction or with atherosclerotic heart disease showed a still higher percentage with elevated extractions.

In an attempt to obtain information concerning the lipoprotein fractions from which the cholesterol was extracted, paper electrophoretic studies were carried out on a) original serum, b) reconstituted lyophilized serum, and c) reconstituted chloroform-extracted, lyophilized serum. Lyophilized sera were reconstituted by the addition of an amount of water equal to the original volume of serum, namely, 0.5 ml. Since the volume occupied by the serum solids was ignored, the concentration of the various substances in these solutions was slightly less than in the original serum.

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Method. A Kelab paper electrophoresis apparatus was employed using Whatman #3 filter paper strips (4.6 x 35 cm) with a barbital-sodium acetate buffer of pH 8.6 and ionic strength .05. Three paper strips separated by plastic strips were used in each of the 2 compartments of the apparatus, thus making a total of 6 strips for each run. To each of the strips at a line 12 cm from the cathode end, 0.05 ml of the test solution was applied. The electrophoresis was run for 15 hours at 5°C unless otherwise stated, with 1.2 ma per strip and a potential of approximately 210 volts. The strips were removed from the apparatus at the end of the electrophoretic procedure and processed as follows: 2 strips were dried at 100°C for 45 minutes, then stained for protein with bromphenol blue according to the method of Köiw, *et al.*(4); 1 strip was air dried and stained for lipids with Sudan black-B; and the remaining 3 strips were kept moist by suspending them in a 1000 ml cylinder containing about half an inch of water and covered with an inverted Petri dish to reduce evaporation. As soon as the position of the proteins along the stained strips was determined, which seldom exceeded 90 minutes from the time the strips were suspended in the cylinder, the wet strips were cut as indicated below, the sections transferred to 25 ml glass-stoppered Erlenmeyer flasks, and approximately 17 ml of a 1:1 mixture of ethyl alcohol and ethyl ether added to each flask. Eight sections were prepared: 1) the albumin area which also included the alpha$_1$-globulins, 2) the area between the al-

bumin + alpha$_1$-globulins and alpha$_2$-globulins, 3) the alpha$_2$-globulins, with the cut being made half way between the alpha$_2$- and beta-globulins, 4) the beta-globulins, 5) a section between the beta-globulins and 5 mm short of the starting point, 6) a section including the area 5 mm on either side of the starting point which usually included some of the gamma-globulins, 7) the remainder of the gamma-globulins, and 8) a 10 mm section towards the end of the strip to serve as a blank. The amount of cholesterol extracted from these sections was determined using a procedure described previously(5).

Results. Effects of lyophilization. The experimental results are shown in Table I. The values for sections 1 and 2 as well as for sections 6 and 7 have been combined. It will be seen that lyophilization invariably increased the amount of cholesterol remaining around the starting point while decreasing that found in the area of the beta-globulins. The effect was most noticeable in those sera with markedly elevated REC values. The REC concentration seems to be of greater significance in this regard than the total cholesterol concentration since, on the whole, the percentage of the total cholesterol which remained around the starting point in the case of the lyophilized sera was greater in Group 3 than in Groups 1 and 2. Each of these groups showed normal total cholesterol concentration but the REC value was quite elevated in the case of Group 3. The percentage of the total cholesterol present in the albumin + alpha$_1$-globulin area was distinctly greater in those subjects with normal REC values than in those with markedly elevated REC concentrations. The rate of movement of the cholesterol associated with albumin and alpha$_1$-globulins was usually not affected by lyophilization. Actually only one of the 21 sera tested showed a definite drop in the cholesterol content of the albumin + alpha$_1$-globulin area following lyophilization. Apparently lyophilization affects primarily the migration of the cholesterol normally present in the general area of the beta-globulins.

Extractability of the lyophilized serum by cold chloroform: Experimental data on the effect of chloroform extraction on the cholesterol distribution are shown in Table II. The values from sections 1-3 have been combined and also those of sections 6 and 7. The sera studied fall into 3 main groups: a) those with normal REC values, b) those with fairly elevated REC values and c) those from which the cold chloroform extracted a large percentage of the total cholesterol. It will be seen that in those with normal REC concentrations most of the cholesterol extracted came from around the starting point. When the REC concentration was very high most of the cholesterol between the beta-globulins and starting point was removed. In 2 cases with extremely high extraction values, no demonstrable cholesterol was left in this area.

Discussion. Previous work has shown that lyophilization, even when followed by cold chloroform extraction, caused no demonstrable change in the migration of the main serum proteins as determined by the bromphenol blue technic(6). The beta-lipoproteins apparently constitute such a small portion of the beta-globulins that a change in their rate of migration is not demonstrated by the bromphenol blue technic. Hunter(7) found no bromphenol blue staining in the area giving the strongest staining with osmium tetraoxide vapors. The ratio of the cholesterol present in the albumin + alpha-globulin area to that present in the remainder of the strip in the 6 normal sera was the same as found by Oliver and Boyd(8) in normal subjects, namely, 28:72. The ratio for all subjects with elevated REC values was invariably less than this.

The fact that the cold chloroform usually removed little cholesterol from the albumin + alpha$_1$- and alpha$_2$-globulin areas can probably be explained by the greater stability of alpha-lipoproteins. Gurd, *et al.*(9) showed that alpha-lipoproteins are stable to lyophilization but purified beta-lipoproteins are not. Since the lyophilized serum in all cases still contained lipoproteins which migrated in the general area of the beta-globulins it would appear that some of the beta-lipoproteins are not denatured by lyophilization. The higher the REC value the greater was the percentage of total cholesterol which remained around the starting point. Swahn(10) has shown

TABLE I. Effect of Lyophilization on Electrophoretic Migration of Serum Cholesterol.

Specimen	Group & No. of subjects	% of total cholesterol in sections 1, 2	3	4	5	6, 7	REC* (range), mg %	Total chol. (range), mg %
Sera from young normal male subjects	1 (6)	21.7 (16.0–31.4)†	6.1 (4.4– 8.2)	37.8 (26.8–52.6)	29.3 (14.6–40.8)	5.0 (1.1–10.3)	16– 35	179–275
Same lyophilized		20.3 (16.2–22.8)	5.7 (2.0–11.6)	20.1 (6.2–27.6)	35.5 (24.5–43.6)	18.3 (13.6–25.1)		
Sera from patients with normal REC & total chol.	2 (7)	14.7 (10.8–14.6)	8.2 (2.8–17.0)	48.1 (40.4–51.7)	22.4 (11.8–33.3)	6.7 (4.7–10.3)	15– 35	141–291
Same lyophilized		13.8 (9.0–20.9)	6.4 (5.8– 7.0)	22.8 (14.1–29.1)	30.9 (19.4–39.7)	26.0 (17.5–33.1)		
Sera from patients with elevated REC & normal total chol.	3 (3)	8.3 (5.9– 9.8)	8.3 (2.7–14.1)	52.4 (51.5–54.1)	25.7 (23.7–28.7)	5.3 (4.7– 5.8)	141–180	256–269
Same lyophilized		8.6 (7.9– 9.2)	4.9 (4.0– 6.5)	22.2 (19.2–24.2)	31.9 (30.8–33.1)	32.3 (29.1–35.0)		
Sera from patients with elevated REC & total chol.	4 (4)	10.7 (7.7–11.2)	3.6 (2.0– 4.8)	46.5 (37.5–56.5)	32.6 (26.8–41.1)	6.7 (3.4–11.2)	107–240	353–430
Same lyophilized		9.6 (8.8–10.7)	3.2 (2.6– 3.7)	11.4 (7.7–16.0)	38.5 (33.8–43.5)	37.3 (36.9–40.7)		
Serum from patient with markedly elevated REC & total chol.	5 (1)	3.5	10.6	64.5	15.9	5.5	670	670
Same lyophilized		4.6	2.8	8.1	12.3	72.1		

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Range in parentheses.

TABLE II. Cholesterol Distribution after Electrophoresis of Lyophilized Sera before and after Chloroform Extraction.

Sample source	Group & No. of subjects	Sections 1, 2, 3	4	5	6, 7	Sum of section values	REC* (range)
		mg %					
Normal subjects	1 (5)	50 (30–66)‡	36 (14– 62)	72 (44–101)	39 (25– 61)	197	16– 35
Residue†		49 (37–64)	30 (18– 37)	72 (57– 88)	22 (10– 34)	173	
Patients with normal REC & total chol.	2 (7)	45 (33–55)	51 (32– 74)	71 (41–109)	58 (29– 76)	225	20– 36
Residue		43 (22–50)	45 (27– 56)	61 (39– 92)	41 (22– 67)	190	
Patients with moderately elevated REC	3 (7)	45 (27–61)	62 (29–101)	113 (85–151)	94 (66–130)	314	66–141
Residue		48 (33–69)	52 (38– 74)	76 (53– 89)	55 (26– 80)	231	
Patients with markedly elevated REC & total chol.	4 (5)	55 (42–90)	74 (48–117)	135 (96–185)	250 (141–451)	514	240–833
Residue		36 (26–47)	25 (7– 55)	26 (5– 53)	17 (0– 51)	104	

* Readily extractable cholesterol—that which cold chloroform extracts from lyophilized serum. † Lyophilized sera after extraction with cold chloroform. ‡ Range in parentheses.

that in paper electrophoresis, chylomicrons remained around the starting point while lipomicrons migrated with the beta-globulins. Since lipomicrons would probably be denatured by lyophilization it is possible that the increase in the cholesterol remaining around the starting point in the lyophilized sera may represent denatured lipomicrons.

The results which have been presented suggest that the cholesterol which is extracted by cold chloroform from lyophilized serum is primarily that present as a constituent of lipo- and chylomicrons. Additional work, however, is necessary to establish this. It is interesting that Albrink, Man and Peters(11) found that the neutral fat which was removed by centrifugation at 18,000 rpm for 1 hour from clear and lactescent sera having total cholesterol values above 300 mg per 100 ml varied from 16.8 to 478 mEq per liter while the amount remaining in the subnatant varied only from 10 to 34 mEq per liter. They assume that the lipids which rose to the surface ranged from 0.1 μ in diameter, the size just sufficient to scatter light(12) to 1 μ, the size of larger chylomicrons. Any cholesterol associated with this material undoubtedly would be included in our REC fraction.

Summary. Lyophilization of serum has been shown markedly to retard electrophoretic migration of some of the cholesterol normally associated with the beta-globulin area but to exert little effect on the cholesterol associated with the albumin + alpha$_1$-globulins. The cholesterol which is extracted by cold chloroform from lyophilized serum comes primarily from that fraction normally found in the beta-globulin area or which remained around the starting point. It is suggested that the cholesterol thus removed represents primarily cholesterol which is associated with lipo- and chylomicrons.

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Coagulation of Salivary Mucoid by Freezing and Thawing of Saliva.* (22329)

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Salivary mucoid,† especially when freshly obtained, may be readily precipitated in the form of a coagulum by the addition of dilute acid to the mucoid-containing fluid(2-4). This characteristic has been used to identify and to prepare mucoid of salivary gland origin(2-5).

It has now been observed that freezing of chemically untreated mucoid-containing salivary secretions for varied periods of time re-

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† The terminology suggested by Meyer(1) is used throughout this report.