New method for partial delipidization of serum lipoproteins

ANDERS GUSTAFSON*

Cardiovascular Section, Oklahoma Medical Research Institute, Oklahoma City, Oklahoma

SUMMARY A new method of partial delipidization of serum lipoproteins has been developed which removes neutral lipids preferentially and yields phospholipid–protein residues in soluble form without the use of detergents. Purified serum lipoprotein fractions were lyophilized in the presence of insoluble starch, which protected the proteins against damage during freezing, and the dry lipoproteins were partially delipidized by consecutive extractions with *n*-heptane. All neutral lipids and some phospholipids, mainly lecithin, were removed. Soluble phospholipid–protein residues were recovered by extraction with buffer solutions in yields ranging from 50 to 90%.

The characteristic differences in the phospholipid–protein ratios between individual lipoprotein fractions are probably determined by the binding properties, dependent on primary structure, of the corresponding protein moieties.

KEY WORDS lipoproteins · high-density · low-density · very low-density · human serum · partial delipidization · lyophilization · insoluble starch · *n*-heptane · neutral lipids · phospholipids

SOLVENT EXTRACTION of serum lipoproteins has frequently been utilized in studies of the chemical nature of their protein moieties and of the nature of the binding forces between lipids and proteins (1).

Complete extraction of lipids can be achieved only after the removal of water molecules which are essential to the structural integrity of the lipoproteins (2). Dehydration can be accomplished either by addition of methanol or ethanol or by freeze-drying the lipoprotein solutions.

The extraction of α-lipoproteins by an alcohol–diethyl ether mixture at low temperature (3) resulted in a water-soluble, lipid-free protein with an unimpaired recombining capacity toward lipids (4). Application of the same procedure for the extraction of β-lipoproteins (5) and VLD-lipoproteins (6), however, yielded protein residues that were insoluble in water.

Dehydration by freeze-drying procedures (7,8) caused denaturation of serum lipoproteins. However, it has been shown that glycerol, glucose, and sucrose will partially protect lipid–protein complexes, including β-lipoproteins, against denaturation by freezing at $-37°$ (9). To protect the plasma clearing factor during its purification by substrate complex formation (10), a mixture of plasma and artificial fat emulsion was lyophilized in the presence of insoluble starch.

The extraction of serum lipoproteins by nonpolar organic solvents without prior dehydration results in only partially delipidized products. The procedure of McFarlane (11), employing diethyl ether as solvent, has been modified and used under different conditions (5, 8, 12, 13, 14) for the preparation of partially delipidized lipoproteins. These studies have indicated that neutral lipids were extracted in preference to phospholipids and that a low yield of water-soluble lipid–protein residues was accompanied by the formation of an emulsion phase (5, 8) containing residual water-insoluble protein and various lipids. The latter consisted primarily of phospholipids.

The extractability of lipids by nonpolar solvents can be increased by the addition of soaps (15) or detergents (16, 17). Treatment of α- and β-lipoproteins with diethyl ether in the presence of detergents results in nearly quantitative removal of triglycerides and cholesterol, but only partial removal of phospholipids. It has been suggested that the detergent forms a detergent–lipo-

Abbreviations: S_f, flotation rate, expressed in Svedberg units (10^{-13} g-cm/dyne sec) of flotation in NaCl medium, density 1.063 g/ml at 26°; $S_f°$, flotation rate at infinite dilution; VLD-lipoproteins, very low-density lipoproteins, d 1.006 and $S_f > 20$; α-lipoproteins, high-density lipoproteins, d 1.063–1.21 g/ml; β-lipoproteins, low-density lipoproteins, d 1.006–1.063 and S_f 0–20.

* Present address: Sahlgrenska sjukhuset, The University of Göteborg, Göteborg, Sweden.

protein micelle (16), thus allowing a deeper penetration of diethyl ether and a more complete extraction of lipids.

Since the partial delipidization of serum lipoproteins by organic solvents has been established as a valuable technique for studying the lipoprotein structure as well as the nature of the bonds between lipids and proteins, the development of a method which would provide improved yields of water-soluble lipid–protein residues of reproducible composition would be of value. In order to remove neutral lipids preferentially and to preserve the phospholipid–protein residues in soluble form without the use of detergents, serum lipoproteins have been lyophilized in the presence of starch as a protective agent and extracted by the nonpolar solvent *n*-heptane (18). The present communication describes the application of this new method for the partial delipidization of α- and β-lipoproteins, and various subfractions of VLD-lipoproteins, and the chemical composition of the corresponding lipid–protein residues.

METHODS

Blood samples were obtained from fasted healthy subjects for the isolation of α- and β-lipoproteins and from patients with hyperglyceridemia for the isolation of VLD-lipoproteins. The blood was allowed to clot and serum was recovered after low speed centrifugation.

Isolation of Lipoproteins

Serum lipoproteins were obtained by sequential preparative ultracentrifugation carried out in the No. 40 rotor of the Spinco Model L ultracentrifuge at 4°. Five VLD-lipoprotein subfractions were isolated at density 1.0055 g/ml utilizing successively increased speeds and times of ultracentrifugation (19). The buffer solution (1.42 g of Na_2HPO_4, 7.27 g of NaCl and 0.1 g of disodium EDTA per liter), density 1.0055 g/ml at 25°, was adjusted to pH 7.0 with N HCl. Fraction A (S_f >5000) was isolated as the top layer after centrifugation at 10,000 × *g* for 10 min, fraction B (S_f 400–5,000) at 20,000 × *g* for 30 min, fraction C (S_f 100–400) at 80,000 × *g* for 60 min, fraction D (S_f 50–100) at 105,000 × *g* for 2 hr, and fraction E (S_f 20–50) at 105,000 × *g* for 22 hr. After removal of VLD-lipoproteins at density 1.019 g/ml, β- and α-lipoproteins were isolated at densities 1.063 g/ml and 1.210 g/ml, respectively, according to the technique described by Bragdon, Havel, and Boyle (20). Each lipoprotein fraction was recentrifuged three times under identical conditions to remove contaminating proteins, including albumin. The repeated centrifugations resulted also in a desirable concentration of the lipoprotein preparations.

Electrophoresis

The course of the purification of lipoprotein fractions was followed by electrophoresis on cellulose acetate or starch gel. The former was performed according to the technique described by Kohn (21) employing barbital buffer, pH 8.6, ionic strength 0.075, and the latter by the technique of Poulik and Smithies (22) utilizing a discontinuous system of buffers (23).

Thin-Layer Chromatography

Glass plates (20 × 20 cm) were coated with Silica Gel G (E. Merck, Darmstadt, West Germany) by use of a Desaga applicator (24). Plates were developed with petroleum ether–diethyl ether 95:5 (v/v) and the spots detected by spraying with 50% (v/v) sulfuric acid and subsequent charring.

Partial Delipidization by n-Heptane

Lipoprotein fractions were dialyzed for 48 hr at 4° against four changes of distilled water containing 0.01% disodium EDTA, pH 7.0. To each milliliter of lipoprotein solution, containing approximately 0.25% of protein, 100 mg of insoluble, purified potato starch powder (J. T. Baker Chemical Company, Phillipsburg, N. J.) were added by stirring. Portions of such mixtures, containing 5–25 mg of lipoprotein protein, were quickly frozen in an acetone–dry ice bath at −70°, lyophilized for 6–8 hr, and stored in a desiccator over P_2O_5 at 4°. Samples were stored in this manner for as long as 4 months without any apparent changes.

Partial delipidization of lyophilized lipoprotein fractions was accomplished by several extractions with *n*-heptane at −12°. During each extraction the mixture of dried lipoprotein and starch was shaken vigorously with 40 ml of *n*-heptane (spectranalyzed grade, Fisher Scientific Company, Houston, Tex.). The first extraction was performed for 60 min and each additional extraction for 20 min. After each treatment with solvent the lipoprotein–starch mixture was sedimented by low speed centrifugation for 10 min at 4°, the *n*-heptane decanted and fresh solvent added. The heptane extracts were evaporated under nitrogen and reduced pressure and the residues monitored by thin-layer chromatography for the presence of sterols, sterol esters, triglycerides, and phospholipids. The absence of neutral lipids in heptane extracts was the main criterion for discontinuing further delipidization. The number of extractions varied from one sample to another and depended primarily on the amount of lipoprotein protein. Samples containing less than 10 mg of lipoprotein protein usually required five extractions. Larger samples required up to 15 consecutive extractions with heptane before they were free of neutral lipids.

The final sediment was dried under nitrogen for 30 min at 4° to remove the last traces of heptane, extracted with 4 volumes of 0.05 M phosphate buffer, pH 7.0, or 0.3 M borate buffer, pH 8.2, or 0.15 M NaCl at 4°, and the soluble lipid–protein residue was separated from the starch by low speed centrifugation. The dried mixture of lipid–protein residue and starch could be stored for several days without any apparent change, but the physical–chemical characterization of phospholipid-protein residues was performed within 48 hr after extraction.

Lipid Analyses

For complete delipidization, the lipoprotein fractions and the lipid–protein residues were extracted with chloroform–methanol 2:1 (v/v). The lipid extracts were washed with 0.58% NaCl solution, the two phases allowed to separate overnight, and the chloroform layer dried with anhydrous Na_2SO_4. Total lipid content was determined gravimetrically in portions of the chloroform extract evaporated under nitrogen and dried to constant weight over P_2O_5 in a vacuum desiccator. Cholesterol was determined according to the method of Sperry and Webb (25), lipid phosphorus by the wet digestion procedure of Youngburg and Youngburg (26), with phosphate measurement by the method of Fiske and Subbarow (27), and triglycerides by the method of Van Handel and Zilversmit (28). The factor 25 was used to convert lipid phosphorus to phospholipids.

Protein Analyses

Protein was determined by a slightly modified method of Lowry, Rosebrough, Farr, and Randall (29). Turbidity caused by high concentration of lipids was removed by extraction with diethyl ether at room temperature after the color had fully developed.

RESULTS

Partial Delipidization of Lyophilized Lipoprotein Fractions by n-Heptane

The extraction of lyophilized lipoprotein fractions by *n*-heptane in the presence of starch resulted in a complete removal of neutral lipids and a variable removal of phospholipids (Table 1). The main phospholipid extracted was lecithin. The product of the partial delipidization consisted of soluble residues characterized by phospholipid/protein ratios varying from 1.6 for the S_f >5000 fraction to 0.8 for the α-lipoproteins. There was a remarkable difference in the extractability of phospholipids from various classes of lipoproteins: less than 5% of phospholipids could be removed from α- and β-lipoproteins but almost 65% from VLD-lipoprotein fractions. Nevertheless, the phospholipid/protein ratios of VLD-lipoprotein residues retained relatively high values. The recovery of soluble residues obtained by delipidization with *n*-heptane was relatively high for most lipoprotein fractions (Table 1). The only exception was a very low yield of soluble residue from β-lipoproteins. The low yield could be increased to 50% by using a buffer containing 2 M urea for the extraction.

TABLE 1 COMPOSITION OF PHOSPHOLIPID–PROTEIN RESIDUES OBTAINED BY PARTIAL DELIPIDIZATION OF LIPOPROTEIN FRACTIONS BY *n*-HEPTANE; AND THE PERCENTAGE RECOVERY OF PROTEIN

Lipoprotein Fraction	Phospholipid/Protein Ratio		Neutral Lipids Extracted by *n*-Heptane	Phospholipid Extracted by *n*-Heptane	Recovery of Soluble Protein
	Lipoprotein	Soluble Residue			
	mg/mg	*mg/mg*	%	%	%
S_f > 5000	4.7	1.6	100.3	—	53
S_f 400–5000	3.8	1.4	100.2	—	48
S_f > 400	3.5	1.3	100.0	65.3	93
S_f 100–400	2.6	1.3	99.9	53.7	93
S_f 50–100	1.4	1.2	100.0	—	44
S_f 20–400	1.8	1.2	99.2	48.8	60
β	0.8	0.7	99.8	1.5	10
α	0.9	0.8	100.0	4.2	100

The sequential removal of neutral lipids and phospholipids by consecutive extractions of lipoprotein fractions with *n*-heptane was followed routinely by thin-layer chromatography and by the quantitative determination of total cholesterol, triglycerides, and phospholipids in corresponding extracts. The results of these procedures applied to the S_f 20-400 fraction are presented in Fig. 1 and Table 2. The major portion of cholesterol, cholesterol esters, and triglycerides was removed by the first two extractions and only traces of these lipids were detected in following steps (Fig. 1, extracts 1–5). The complete extraction of phospholipid–protein residue showed the presence of only one spot, corresponding to phospholipids (Fig. 1, extract 6). Thin-layer chromatography revealed an interesting difference in the extractability of free and esterified cholesterol from β- and α-lipoproteins. Free cholesterol was more tightly bound than cholesterol esters in β-lipoproteins and less so in α-lipoproteins.

In contrast to neutral lipids, the removal of phospholipids appeared to be a slow and continuous process. The extraction of residues free from neutral lipids resulted in a further removal of small amounts of phospholipids (Table 2). To obtain residues of constant phospholipid/protein ratios the delipidization procedure was terminated after the last traces of cholesterol and triglycerides had been removed. Otherwise, the continued extraction of phospholipid resulted in a diminishing phospholipid/protein ratio and yield of soluble protein. The number of consecutive extractions required depended on the total amount of protein in the original

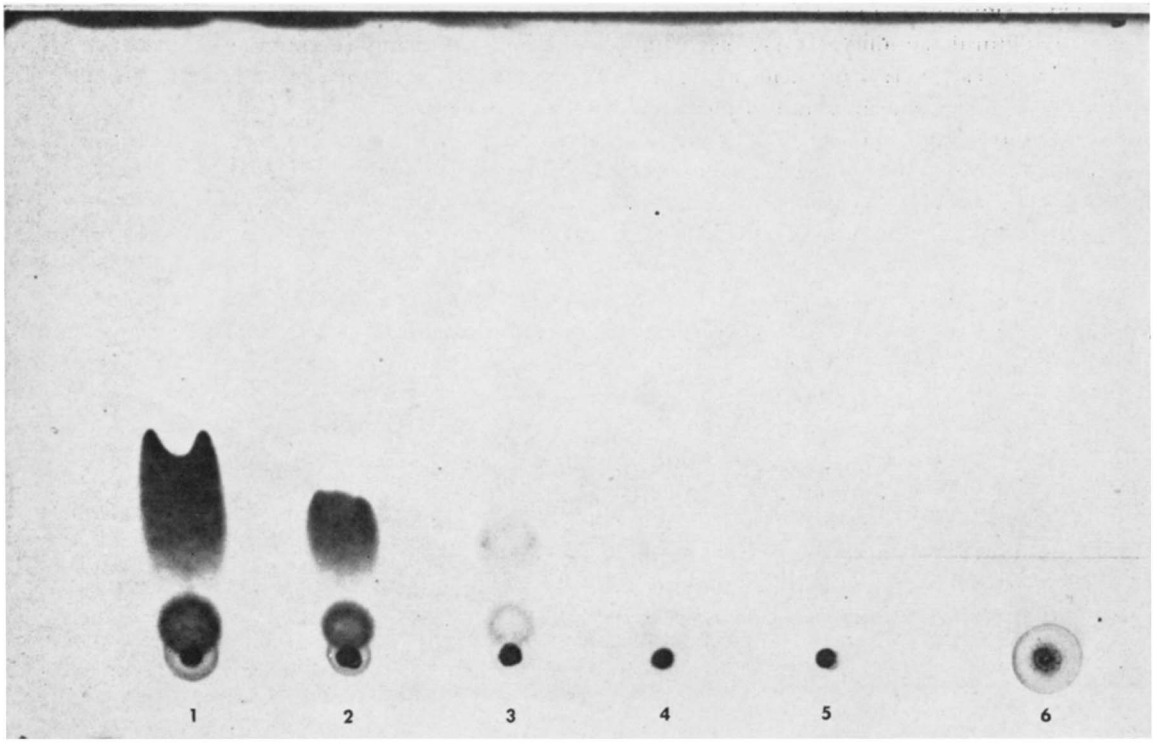

FIG. 1. Thin-layer chromatography of lipid extracts from the VLD-lipoprotein fraction, S_f 20–400. *1–5*, five consecutive heptane extracts of the lipoprotein–starch mixture. *6*, chloroform–methanol extract of the phospholipid–protein residue.

lipoprotein fraction. An example of reproducibility of the method applied to the VLD-lipoprotein fractions is shown in Table 3.

DISCUSSION

Anfinsen and Quigley (10) have shown that by lyophilization of an enzyme–substrate complex consisting of lipoprotein lipase and coconut oil emulsion, and by subsequent extraction of lipids by *n*-hexane in the presence of starch, an active enzyme preparation of a high degree of purity could be obtained. In the present study a modification of this procedure has been successfully applied for partial delipidization of serum lipoprotein fractions. Lovelock (9) has shown that during freezing, factors such as changes in pH, salt concentration, and osmotic pressure of the media caused denaturation of the lipoproteins of egg yolk and cell membranes. On the other hand, denaturation of serum β-lipoproteins coincided directly with the removal of the last traces of water from the medium. Such irreversible damage could be explained either by assuming that water constitutes an essential part of the lipoprotein structure (8, 30) or that water serves as a protective coating that prevents collisions between lipoprotein complexes (9). In either case water takes part in various types of associations with the protein and lipid moieties within or be-

TABLE 2 SEQUENTIAL REMOVAL OF LIPIDS BY PARTIAL DELIPIDIZATION OF S_f 20–400 VLD-LIPROPROTEIN FRACTION BY *n*-HEPTANE

	Cholesterol		Phospholipid		Triglyceride	
	mg	%*	*mg*	%*	*mg*	%*
First extraction	11.38	92.6	2.54	57.7	93.00	77.4
Second "	0.85	6.9	0.46	10.6	25.50	21.2
Third "	0.06	0.5	0.48	11.0	0.71	0.6
Fourth "	0.01	0.0	0.67	15.2	0.56	0.5
Fifth "	0.00	0.0	0.24	5.5	0.30	0.3
Total extracted	12.30	100.0	4.39	100.0	120.07	100.0

* Per cent lipid extracted by *n*-heptane.

TABLE 3 REPRODUCIBILITY OF THE *n*-HEPTANE DELIPIDIZATION PROCEDURE

	Phospho-lipid	Neutral Lipids	Protein	Phospho-lipid/ Protein Ratio
	mg	*mg*	*mg*	*mg/mg*
S_f 100–400 fraction				
Phospholipid–Protein Residue I	3.7	0	3.4	1.11
Phospholipid–Protein Residue II	3.5	0	3.4	1.03
$S_f > 20$ fraction				
Heptane, Extract I	5.9	115.3	0	—
Heptane, Extract II	6.3	117.5	0	—

tween lipoprotein complexes and contributes significantly to their structural stability. It has been found that compounds such as glucose, glycerol, and sucrose (9) can substitute for water molecules and protect serum and membrane lipoproteins against denaturation by freezing. It appears from the present delipidization experiments that starch, a water-insoluble compound rich in hydroxyl groups, offers essentially the same protection to lipoproteins as water-soluble carbohydrates. Moreover, starch has an additional advantage over water-soluble carbohydrates in that the phospholipid–protein residues can be easily separated from the insoluble starch.

So far, partial delipidization of purified lipoprotein fractions by relatively nonpolar solvents has been conducted only in aqueous solutions, with the possible exception of an attempt by Forbes, Forbes, and Petterson (31) to extract lyophilized whole serum by chloroform. However, only a partial removal of cholesterol was achieved, possibly because of the presence of serum proteins which prevented solution of the lipid. It has been shown recently (32) that the addition of 5% albumin was very effective in decreasing the extraction of cholesterol from lipoprotein solutions shaken with *n*-heptane.

Our attempts to submit lipoprotein fractions to extraction by diethyl ether in a biphasic system resulted in an incomplete removal of neutral lipids and low yields of soluble residues. In general, residues obtained by diethyl ether extraction of most lipoprotein fractions had lower phospholipid/protein ratios than those prepared from the same fractions by extraction with *n*-heptane. A lower phospholipid content would explain the increased susceptibility to precipitation. Most of these difficulties have been successfully circumvented by extraction of a mixture of dried lipoproteins and starch with *n*-heptane. By increasing the surface area and exposing the lipoproteins in a dry state to closer contact with and deeper penetration by the solvent this procedure results in the complete removal of neutral lipids and partial removal of phospholipids. The great importance of the contact area between solvent and lipid–protein complexes has been demonstrated recently in an independent study (32) in which the extraction of cholesterol from lipoprotein monolayers spread between phosphate buffer and heptane varied from 54 to 100% depending on the completeness of lipoprotein spreading.

It is of interest that a considerably higher percentage (50–65%) of phospholipid can be extracted from VLD-lipoprotein than from α- and β- lipoproteins. Nevertheless, residues obtained from VLD-lipoproteins had relatively high phospholipid/protein ratios. These findings, in addition to supporting the earlier conclusions that phospholipids are attached to proteins by stronger bonds than neutral lipids, suggest that the difference in the amount of firmly bound phospholipids is probably determined by the characteristic primary structure of each protein moiety.

Application of this procedure for the partial delipidization of VLD-lipoproteins ($S_f > 20$) has resulted in the isolation of three distinct phospholipid–protein residues characterized ultracentrifugally as 4S, 14S, and 7S (33). The 4S and 14S residues are similar to those obtained from α- and β-lipoproteins, respectively. The chemical characterization of all of these phospholipid–protein complexes will be presented in a separate communication.

The author, a Cardiovascular Research Trainee supported by U. S. Public Health Service Grant HTS-5403, wishes to express his gratitude to Dr. R. H. Furman and Dr. P. Alaupovic for valuable discussions, advice, and criticisms, to Mr. M. Sullivan and Mrs. S. Walraven for capable technical assistance, and to Mrs. M. Farmer for assistance in the preparation and typing of the manuscript.

Manuscript received March 4, 1965; accepted June 28, 1965.

References

1. Gurd, F. R. N. In *Lipide Chemistry*, edited by D. J. Hanahan. John Wiley, New York, 1960, pp. 260–325.
2. Dervichian, D. G. *Discussions Faraday Soc.* **6:** 7, 1949.
3. Scanu, A., L. A. Lewis, and F. M. Bumpus. *Arch. Biochem. Biophys.* **74:** 390, 1958.
4. Scanu, A., and W. L. Hughes. *J. Biol. Chem.* **235:** 2876, 1960.
5. Scanu, A., L. A. Lewis, and I. H. Page. *J. Exptl. Med.* **108:** 185, 1958.
6. Scanu, A., and I. H. Page. *J. Exptl. Med.* **109:** 239, 1959.
7. Gurd, F. R. N., J. L. Oncley, J. T. Edsall, and E. J. Cohn. *Discussions Faraday Soc.* **6:** 70, 1949.
8. Oncley, J. L., F. R. N. Gurd, and M. Melin. *J. Am. Chem. Soc.* **72:** 458, 1950.
9. Lovelock, J. E. *Proc. Roy. Soc. London, Ser. B* **147:** 427, 1957.
10. Anfinsen, C. B., Jr., and T. W. Quigley, Jr. *Circulation* **8:** 435, 1953.
11. McFarlane, A. S. *Nature* **149:** 439, 1942.
12. Avigan, J. *J. Biol. Chem.* **226:** 957, 1957.
13. Grundy, S., H. L. Dobson, and A. C. Griffin. *Proc. Soc. Exptl. Biol. Med.* **100:** 704, 1959.
14. Hayashi, S., F. Lindgren, and A. Nichols. *J. Am. Chem. Soc.* **81:** 3793, 1959.
15. Macheboeuf, M., and F. Tayeau. *Bull. Soc. Chim. Biol.* **23:** 49, 1941.
16. Ayrault-Jarrier, M. *Bull. Soc. Chim. Biol.* **43:** 153, 1961.
17. Ayrault-Jarrier, M., F. Martinet, and J. Polonovski. *Bull. Soc. Chim. Biol.* **45:** 369, 1963.
18. Gustafson, A. *Biochim. Biophys. Acta* **84:** 223, 1964.
19. Gustafson, A., P. Alaupovic, and R. H. Furman. *Biochemistry* **4:** 596, 1965.
20. Bragdon, J. H., R. J. Havel, and E. Boyle. *J. Lab. Clin. Med.* **48:** 36, 1956.
21. Kohn, J. *Clin. Chim. Acta* **3:** 450, 1958.

22. Poulik, M. D., and O. Smithies. *Biochem. J.* **68:** 636, 1958.
23. Poulik, M. D. *Nature* **180:** 487, 1957.
24. Stahl, E. In *Dünnschicht-Chromatographie*, edited by E. Stahl. Springer-Verlag, Berlin, 1962, p. 5.
25. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
26. Youngburg, G. E., and M. V. Youngburg. *J. Lab. Clin. Med.* **16:** 158, 1931.
27. Fiske, C. H., and Y. Subbarow. *J. Biol. Chem.* **66:** 375, 1925.
28. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50:** 152, 1957.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* **193:** 265, 1951.
30. Vandenheuvel, F. A. *Can. J. Biochem. Physiol.* **40:** 1299, 1962.
31. Forbes, J. C., A. L. Forbes, and O. M. Petterson. *Proc. Soc. Exptl. Biol. Med.* **105:** 628, 1960.
32. Zilversmit, D. B. *J. Lipid Res.* **5:** 300, 1964.
33. Gustafson, A., P. Alaupovic, and R. H. Furman. *Biochim. Biophys. Acta* **84:** 767, 1964.