Purification and characterization of bovine lipoproteins: resolution of high density and low density lipoproteins using heparin-Sepharose chromatography

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Abstract The selective and reversible adsorption of bovine low density lipoproteins (LDL) by heparin-Sepharose has been exploited as the critical step in a procedure for the preparative isolation of very low density lipoproteins (VLDL)/chylomicrons, LDL, and high density lipoproteins (HDL) from bovine plasma. Molecular size exclusion chromatography and isopycnic density gradient separation steps are also involved in the method described. The resulting HDL and LDL fractions are free from contamination by one another as judged by electrophoretic mobility in agarose gels. The major lipid and apolipoprotein compositions of the three resolved lipoprotein classes have been determined. —**Cordle, S. R., R. A. Clegg, and S. J. Yeaman.** Purification and characterization of bovine lipoproteins: resolution of high density and low density lipoproteins using heparin-Sepharose chromatography. *J. Lipid Res.* 1985. **26:** 721–725.

Supplementary key words apoprotein composition

The resolution of three major populations of plasma lipoprotein by sequential collection of fractions floating between defined solvent density limits was originally devised for application to human plasma (1, 2). Numerous similar fractionation procedures have been developed subsequently for the lipoproteins of other species of animals, although it should be noted that the appropriate density limits (i.e., those resulting in populations of particles that are mutually distinct on the basis of size, electrophoretic properties, and compositional criteria) may not be the same as those applicable to human lipoproteins; the rat affords the best-studied example of this (e.g., ref. 3).

In the case of bovine lipoproteins, no entirely satisfactory density 'window' for the quantitative isolation of LDL has been identified. Because of the much greater relative abundance of bovine HDL (83% by weight of total serum lipoprotein, compared to 31% in man, (4)) and because of its rather low mean bouyant density (5, 6), bovine LDL preparations fractionated on the basis of bouyant density alone always contain some particles with HDL-like properties (e.g., presence of apoA-I, and α-electrophoretic mobility as reported in (7)). Homogeneous lipoprotein preparations are essential for studies aimed at elucidating their metabolic roles (8). However, only one other group has reported an alternative strategy for the preparative isolation of bovine lipoproteins (9) and their chosen method of agarose gel chromatography did not yield homogeneous lipoprotein fractions (9, 10).

The ability of heparin-Sepharose to bind β-lipoproteins at low salt concentrations and the role of their apolipoproteins in this interaction have been described by Iverius (11) and have been exploited to separate VLDL and LDL from the total serum lipoproteins of several species. Additionally, heparin-Sepharose chromatography has been used to fractionate HDL into subclasses, with apoE-rich HDL particles being selectively bound to the immobilized heparin (e.g., 12). Previously published analyses of bovine HDL apoproteins (13–15) have furnished no evidence for the presence of apoE in this species. This apparent absence of apoE from major bovine lipoprotein classes is exploited in the method described here to effect a clean separation between LDL and HDL using heparin-Sepharose chromatography. Bovine LDL prepared by this method was found to be free of α-migrating particles as judged by agarose gel electrophoresis and to band symmetrically in isopycnic KBr gradients at a mean density of 1.05 g/ml.

Abbreviations: VLDL, LDL, and HDL, very low density, low density, and high density lipoproteins as further defined in the text; EDTA, ethylene diamine tetraacetic acid; DTNB, 5,5-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; PEG 6000, polyethylene glycol, average molecular weight 6000.

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MATERIALS AND METHODS

Animals and samples

Whole bovine blood, obtained from a local slaughterhouse, was collected into trisodium citrate to achieve a final concentration of 15 mM. Plasma was separated from cellular elements by centrifugation (2000 *g*, 20 min) at 4°C, and DTNB was added to a final concentration of 1 mM. Samples were then further processed without delay.

Preparation of lipoproteins

Sequential differential flotation in KBr solutions (1) was used to prepare reference rat lipoproteins between the following density limits (3): VLDL, <1.006 g/ml; LDL, 1.006–1.040 g/ml; HDL, 1.063–1.21 g/ml. The preparation of bovine lipoproteins is described in the text. Preparative ultracentrifugation was done in a 60Ti fixed-angle rotor in a Beckman L2/65B centrifuge at 10°C. Isopycnic banding of lipoproteins in KBr gradients was performed according to the method of Chapman et al. (16).

The separations illustrated in the figures are typical examples of at least three that have been performed on different samples.

Analytical methods

Agarose gel electrophoresis was performed as described by Noble (17), except that an 80 mM Tris–24 mM tricine buffer system (pH 8.6) was used throughout. Lipoprotein samples in this buffer were diluted with an equal volume of homologous lipoprotein-free serum prior to electrophoresis. Similarly, serum and plasma samples were diluted with an equal volume of buffer. These steps were necessary in order to ensure comparable electrophoretic migration of individual lipoproteins when analyzed either as a component of unfractionated plasma/serum, or in purified form. SDS-polyacrylamide gel electrophoresis was conducted by the procedure of Laemmli and Favre (18). Quantitation of acylglycerols, phospholipids (as phosphatidylcholine), and free and esterified cholesterol was by standard enzymatic procedures, as described by Christie (19). Major lipid classes were separated by thin-layer chromatography on silica gel (19). Analytical values quoted are means of determinations performed on at least three separate preparations.

Materials

Heparin-Sepharose CL-6B and Sepharose CL-2B were from Pharmacia; enzymes for lipid analysis were from the Boehringer Corporation Ltd. Agarose gels were prepared from Sigma type I agarose. All other chemicals, of analytical purity where possible, were obtained from BDH Ltd.

RESULTS

Separation of VLDL/chylomicrons from LDL and HDL by gel filtration chromatography

Total lipoproteins ($d < 1.21$ g/ml) from bovine plasma were first concentrated to a volume equivalent to approximately 2% of the original plasma by ultracentrifugal flotation and dialysis against 50% (w/v) PEG 6000. The resulting lipoprotein-containing sample was chromatographed at 2°C on a column of Sepharose CL-2B (26 × 640 mm) equilibrated in 100 mM-Tris-HCl, 0.02% NaN_3, pH 8. Quantitative recovery of protein, as monitored by absorbance at 280 nm, was achieved in the two peaks into which the sample was resolved. The first (peak A) contained about 2% of the loaded protein, and emerged in the void volume of the column ($K_{av} = 0$); the second (peak B) was eluted at a K_{av} value of 0.35. The predominant lipoprotein in the opalescent fractions comprising peak A was subsequently shown to resemble VLDL/chylomicrons (see below). Flotation of lipoproteins contained in peak B fractions in buoyant density gradients of KBr revealed the presence of a single peak of protein (absorbance at 280 nm) centered on a density of 1.08 g/ml (not shown). However, agarose gel electrophoretic analysis indicated the presence of both α- and β-migrating lipoproteins in peak B (see Fig. 2), suggesting the presence of both LDL and HDL. Analysis, by this technique, of fractions across the density gradient revealed considerable overlap in the buoyant density characteristics of the two electrophoretically discernible lipoproteins (results not shown); the equilibrium density flotation method was therefore unsuitable for the quantitative separation of LDL from HDL, as previously found by others (5–7, 10, 20, 21).

Resolution of LDL and HDL by heparin-Sepharose chromatography

Peak B from the Sepharose CL-2B was concentrated by dialysis against PEG 6000 as above and loaded onto a column of heparin-Sepharose. Most of the material that absorbed at 280 nm passed unretarded through the column (peak I, **Fig. 1**). Adsorbed material was then eluted as a single peak (peak II, Fig. 1) during development of the column with an NaCl gradient. Agarose gel electrophoretic analysis showed that material in Peak I consisted of lipoproteins with α-mobility, and that in Peak II fractions exhibited β-mobility. There was no evidence, using this technique, of cross-contamination between α- and β-lipoproteins (**Fig. 2**). However, SDS-polyacrylamide gel electrophoretic analysis showed that both peaks contained discernible amounts of albumin. It was the presence of this, and other soluble serum proteins, that necessitated the addition of a further preparative step in which

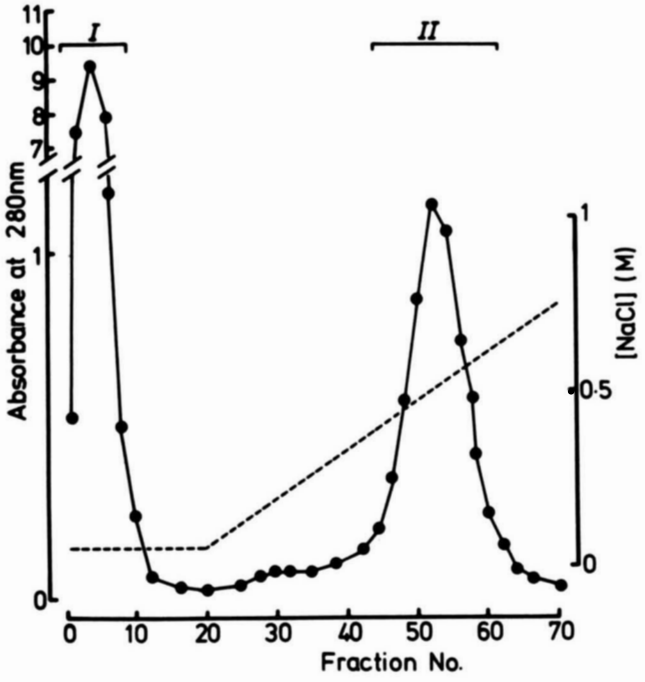

Fig. 1. Resolution of α and β lipoproteins by heparin-Sepharose chromatography. Peak B lipoproteins, concentrated after separation on Sepharose CL-2B (from 180 ml of original plasma) were loaded, in a volume of 4 ml, onto heparin-Sepharose (16 × 120 mm) equilibrated with 2 mM sodium phosphate, 50 mM NaCl, 1 mM EDTA, pH 7.2. This and subsequent operations were done at 4°C. The column was washed with this buffer at a flow rate of 24 ml • hr^{-1} until the A_{280} of the eluate fell to less than 0.05 (approximately two column volumes were typically required). Elution was then continued with a linear gradient (100 ml) from 0.05 to 0.75 M NaCl (- - -) in 2 mM Na-phosphate, 1 mM EDTA, pH 7.2, at the same flow rate. The eluate was collected in 2-ml fractions and monitored for the presence of A_{280}-absorbing material (● — ●). Fractions comprising peaks I and II were pooled as indicated.

the α- and β-lipoproteins, separated by heparin-Sepharose chromatography, were subjected to equilibrium buoyant density centrifugation in gradients of KBr. One major component was obtained in this way in the α-lipoprotein sample, having a mean buoyant density of 1.08 g/ml, and one also in the β-lipoprotein sample, having a density of 1.05 g/ml (**Fig. 3**). These major components accounted for more than 70% of the protein loaded onto the gradient in each case: their buoyant densities justify their classification as HDL and LDL, respectively. An additional zone of lipoprotein of very high apparent density (about 1.26 g/ml) was present in both peaks I and II from heparin-Sepharose (Fig. 3). This protein-rich material consisted essentially of apoA-I and acylglycerols (not shown). It was not further analyzed.

The major lipoprotein in peak A floated at a density of <1.006 g/ml, with a minor band at about 1.14 g/ml (Fig. 3). On agarose gel electrophoresis, the major lipoprotein remained at the origin (not shown) and is therefore subsequently referred to as a chylomicron fraction.

Apoprotein compositon of isolated lipoproteins

Apolipoproteins were analyzed by SDS-polyacrylamide gel electrophoresis (after delipidation of samples when necessary) as shown in **Fig. 4**. Individual apoproteins were identified on the basis of apparent molecular weights similar to the known components of rat and human lipoprotein standards. Chylomicron apoproteins consisted of a major component of M_r 23,000 which may correspond to an unnamed apolipoprotein of rat VLDL that others have also described in Wistar rat apoHDL (22). Also present was a doublet of peptides, the smaller of which co-migrated with apoA-I (M_r 28,000). A minor component of M_r approximately 50,000 was also detected; this may be analogous to apoA-IV, although it was not detectable in gels deliberately overloaded with bovine apoHDL samples (data not shown).

The principal components of bovine LDL were apoB-like, having apparent molecular weights in excess of approximately 200,000. ApoA-I was a minor component of this lipoprotein.

Confirming previous reports (13–15), the apoproteins of bovine HDL comprised apoA-I and several C-apoproteins. ApoE was not detectable in any of these three lipoproteins.

Lipid composition of bovine lipoproteins

The lipid/protein ratios (w/w) of chylomicrons, LDL, and HDL were 11.2, 7.9, and 4.2, respectively. Relative compositions (wt % of total) in terms of the major lipid classes, triacylglycerol, phospholipid, cholesteryl ester, and nonesterified cholesterol were, respectively, 88.5, 1.2, 5.5, and 4.8 in chylomicrons; 4.6, 27.4, 60.6, and 7.4 in LDL; and 0.34, 40.6, 51.2, and 7.9 in HDL.

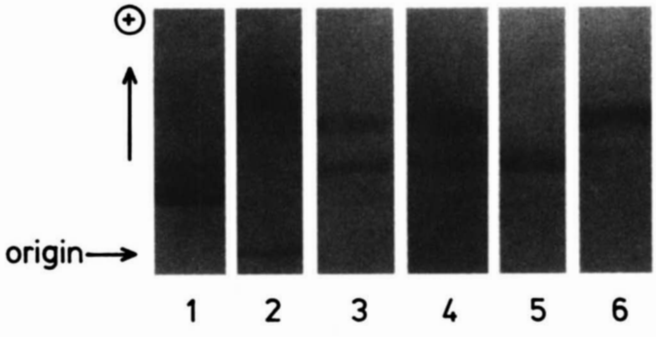

Fig. 2. Agarose gel electrophoretic analysis of bovine lipoproteins. Whole sera and resolved lipoprotein fractions were analyzed by electrophoresis in agarose gels followed by staining with Fat Red. Samples in the indicated lane numbers were as follows: lane 1, human serum; lane 2, rat serum; lane 3, bovine serum; lane 4, peak B from Sepharose CL-2B; lane 5, peak II from heparin-Sepharose (β-migrating); lane 6, peak I from heparin-Sepharose (α-migrating).

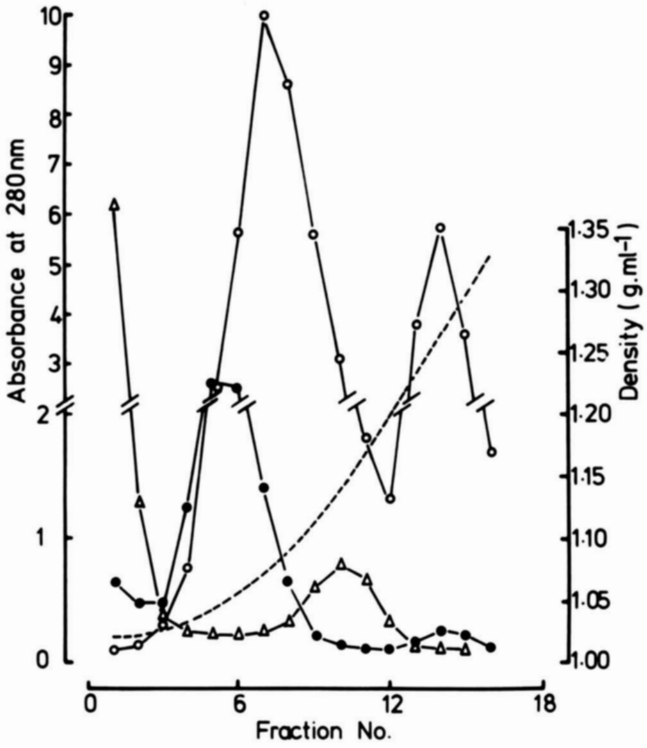

Fig. 3. Buoyant density in KBr gradients of bovine lipoproteins. Peaks I and II from heparin-Sepharose (see Fig. 1) and peak A from Sepharose CL-2B were concentrated by dialysis against PEG 6000, brought to a density of 1.21 g/ml by a further dialysis against 2.48 M KBr, 155 mM NaCl, 1 mM EDTA, 0.02% NaN_3, pH 7.4, and then incorporated into KBr density gradients. These were centrifuged at 40,000 rpm for 48 hr at 15°C in a Beckman SW 40 rotor, and then fractionated and monitored for A_{280} and density, after dilution if necessary. Peak A, (△—△); peak I, (○—○); peak II, (●—●); protein-free density, (- - -).

DISCUSSION

The ability of heparin-Sepharose selectively to bind low density β-lipoproteins from mixtures of bovine LDL and HDL has allowed us to separate these two populations of lipoproteins, between which particle size and density distribution overlap to such an extent that their quantitative separation by more conventional procedures is precluded. Presumably it is because bovine HDL, unlike rat HDL, lacks apoE, as found in this study, that this lipoprotein is not retained by heparin-Sepharose, thereby enabling the method to discriminate effectively between bovine LDL and HDL.

The apoHDL composition determined in this study, while largely confirming previously reported compositions, did not reveal the presence of the M_r 22,000 component found by Ferreri and Gleockler (9) to be the major constituent of bovine HDL. We did find, however, a peptide of this approximate size in chylomicron-apoproteins. None of the lipoproteins characterized in this study contained significant quantities of the M_r 40,000 peptide described by Puppione et al. (6) as the major apoprotein of an IDL fraction (1.006–1.020 g/ml) from lactating cow serum. The physiological roles of some of the low molecular weight apoC peptides in bovine HDL have been elucidated (14, 15, 23). It was unexpected that no apoC peptides were detectable, in the present study, in association with chylomicrons, in view of their function as effectors of lipoprotein lipase. It remains a possibility that the material isolated as chylomicrons consists of remnant particles, having lost some of their original lipid and apoprotein constituents in the course of metabolism in the peripheral tissues. The identity of the apoA-I-rich very high density particles found in this study is uncertain. One possibility is that they may be artefacts induced by the preparation procedures adopted, based on aggregates of apoA-I. Nevertheless, the presence of acylglycerols but not of phospholipids in the particles in question is paradoxical. The lipid compositions of bovine chylomicrons, LDL, and HDL reported here are broadly similar to those found by others (reviewed in ref. 24). The fatty acid compositional analysis of the esterified lipid classes of each lipoprotein (data not shown) confirms the previously reported (24) preponderance of 18:2 and 18:3 in the cholesteryl ester fraction, and of saturated fatty acids (particularly 16:0) in the triacylglycerols.

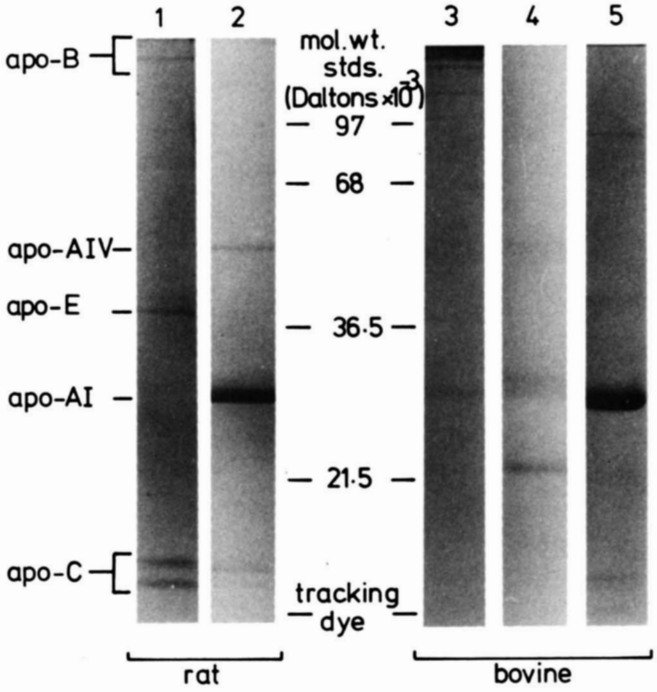

Fig. 4. Apolipoprotein composition of bovine lipoproteins; analysis by SDS-polyacrylamide gel electrophoresis. Coomassie blue-stained 12% SDS-polyacrylamide gels are shown. Samples in the indicated lane numbers were as follows: lane 1, rat LDL; lane 2, rat HDL; lane 3, bovine LDL; lane 4, bovine chylomicrons; lane 5, bovine HDL. The bovine lipoproteins were those purified by the chromatographic and isopycnic flotation techniques described in the text.

The buoyant density profiles of HDL and LDL, separated using heparin-Sepharose, show a marked degree of overlap as anticipated. By discriminating between them using a method sensitive primarily to apoprotein composition, we have achieved the quantitative separation of two populations of lipoprotein particle which are probably metabolically distinct from one another: in any event, the preparative separation which we have described will allow further investigations (W. Koper, S. R. Cordle, and S. J. Yeaman. Submitted for publication.) of the functional roles of LDL and HDL in ruminant lipid metabolism.

S. R. C. was supported by an S.E.R.C. postgraduate R.C.C.A. training award. We are most grateful to Dr. W. W. Christie for advice and interest throughout this work and for help with fatty acid analyses.

Manuscript received 9 October 1984.

REFERENCES

1. Lindgren, F. T. 1975. Preparative ultracentrifugal laboratory procedures and suggestions for lipoprotein analysis. *In* Analysis of Lipids and Lipoproteins. E. G. Perkins, editor. American Oil Chemists' Society, Champaign, IL. 204–224.
2. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
3. Lasser, N. L., P. S. Roheim, D. Edelstein, and H. A. Eder. 1973. Serum lipoproteins of normal and cholesterol-fed rats. *J. Lipid Res.* **14:** 1–8.
4. Mills, G. L., and C.E. Taylaur. 1971. The distribution and composition of serum lipoproteins in eighteen animals. *Comp. Biochem. Physiol.* **40B:** 489–501.
5. Raphael, B. C., P. S. Dimick, and D. L. Puppione. 1973. Lipid characterization of bovine serum lipoproteins throughout lactation and gestation. *J. Dairy Sci.* **56:** 1025–1032.
6. Puppione, D. L., G. M. Forte, A. V. Nichols, and E. H. Strisower. 1970. Partial characterization of serum lipoproteins in the density interval 1.04–1.06 g/ml. *Biochim. Biophys. Acta.* **202:** 392–395.
7. Puppione, D. L., S. T. Kunitake, M. L. Toomey, E. Loh, and V. N. Schumaker. 1982. Physicochemical characterization of ten fractions of bovine alpha lipoproteins. *J. Lipid Res.* **23:** 371–379.
8. Steinberg, D. 1980. Lipoprotein structure and metabolism: inhomogeneity, variability and species specificity. *In* Atherosclerosis, V. A. M. Gotto, L. C. Smith, and B. Allan, editors. Springer-Verlag, New York. 616–623.
9. Ferreri, L. F., and D. H. Gleockler. 1979. Electrophoretic characterization of bovine lipoprotein subfractions isolated by agarose gel chromatography. *J. Dairy Sci.* **62:** 1577–1582.
10. Grummer, R. R., C. L. Davis, and H. M. Hegarty. 1983. Comparison of ultracentrifugation and gel filtration for the isolation of bovine lipoproteins. *Lipids.* **18:** 795–802.
11. Iverius, P. H. 1972. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. *J. Biol. Chem.* **247:** 2607–2613.
12. Quarfordt, S. H., R. S. Jain, L. Lakoi, S. Robinson, and F. Shelburne. 1978. The heterogeneity of rat high-density lipoproteins. *Biochem. Biophys. Res. Commun.* **83:** 786–793.
13. Jonas, A. 1975. Isolation and partial characterization of the major apolipoprotein component of bovine serum high density lipoprotein. *Biochim. Biophys. Acta.* **393:** 460–470.
14. Lim, C. T., and A. M. Scanu. 1976. Apoproteins of bovine serum high density lipoproteins: isolation and characterization of the small-molecular weight components. *Artery.* **2:** 483–496.
15. Clegg, R. A. 1978. Bovine serum apolipoprotein effectors of milk lipoprotein lipase. *Biochem. Soc. Trans.* **6:** 1207–1210.
16. Chapman, M. J., S. Goldstein, D. Lagrange, and P. M. Lapland. 1981. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J. Lipid Res.* **22:** 339–358.
17. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
18. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol.* **80:** 575–599.
19. Christie, W. W. 1982. Lipid Analysis. 2nd edition. Pergamon Press, Oxford.
20. Stead, D., and V. A. Welch. 1975. Lipid composition of bovine serum lipoproteins. *J. Dairy Sci.* **58:** 122–127.
21. Puppione, D. L., S. T. Kunitake, R. L. Hamilton, M. L. Phillips, V. N. Schumaker, and L. D. Davis. 1982. Characterization of unusual intermediate density lipoproteins. *J. Lipid Res.* **23:** 283–290.
22. Connelly, P. W., and A. Kuksis. 1982. SDS-glycerol polyacrylamide gel electrophoresis of plasma apolipoproteins. *Biochim. Biophys. Acta.* **711:** 245–251.
23. Astrup, H. N., and G. Bengtsson. 1982. Activator proteins for lipoprotein lipase from bovine plasma: preparation by adsorption to intralipid. *Comp. Biochem. Physiol.* **72B:** 487–491.
24. Christie, W. W. 1981. The composition, structure and function of lipids in the tissues of ruminant animals. *In* Lipid Metabolism in Ruminant Animals. W. Christie, editor. Pergamon Press, Oxford. 95–191.