Cholesteryl ester-rich microemulsions: stable protein-free analogs of low density lipoproteins

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Abstract A method has been devised for the preparation of stable lipid microemulsions containing cholesterol, cholesteryl ester, phosphatidylcholine, and trioleoylglycerol in the relative molar ratios found in low density lipoproteins. Gel permeation chromatography showed these microemulsions to be essentially homogeneous with respect to chemical composition. Omission of triolein or substitution of a diunsaturated phosphatidylcholine for either a disaturated or monosaturated-monounsaturated phosphatidylcholine destroyed the observed homogeneity of the microemulsions. The particle diameter of the negatively-stained microemulsion averaged 45 nm by electron microscopy. The relative elution volumes of the cholesteryl ester-rich microemulsion, $VLDL_2$, $VLDL_3$, and LDL indicated a mean diameter of about 35 nm. The cholesteryl ester-rich microemulsion can be used as a cholesteryl ester donor for plasma protein-mediated transfer of cholesteryl ester to plasma lipoproteins and for studying apoprotein-lipid interactions.—**Via, D. P., I. F. Craig, G. W. Jacobs, W. B. Van Winkle, S. C. Charlton, A. M. Gotto, Jr., and L. C. Smith**. Cholesteryl ester-rich microemulsions: stable protein-free analogs of low density lipoproteins. *J. Lipid Res.* 1982. **23:** 570–576.

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Low density lipoproteins (LDL) are macromolecular complexes of lipid and protein, and constitute the major source of cholesteryl ester in the bloodstream (1, 2). The components of LDL are believed to be arranged in a micellar structure with a central core of nonpolar triglyceride and cholesteryl ester surronded by a surface shell of phospholipid, cholesterol, and apoproteins (3). Although this general picture of LDL structure is generally accepted, several important structural details remain unresolved. The exact disposition of the lipid components and the precise role of the apoproteins in determining lipoprotein stability are not fully understood. Some previous models for lipid-apoprotein interactions used single bilayer dispersons of lipid (4). However, a more appropriate lipoprotein model for LDL should not have a bilayer structure and should contain cholesteryl esters and triacylglycerols (5). A two-component microemulsion prepared by sonication of egg phosphatidylcholine and cholesteryl oleate has been reported as a possible model for studying apoprotein-lipid interactions (6). Sonicated microemulsions of egg phosphatidylcholine and cholesteryl oleate or egg phosphatidylcholine and trioleoylglycerol have also been made (7). Additionally, a stable emulsion of phosphatidylcholine, cholesterol, and trioleoylglycerol in the approximate ratios found in VLDL has also been formed by sonication (8) and by an injection technique.[1] In this report we describe the preparation and characterization of a stable protein-free micellar cholesteryl ester-rich microemulsion containing the four major lipid components of LDL in molar proportions similar to those found in the native lipoprotein.

MATERIALS AND METHODS

Preparation of microemulsions

Separate solutions containing 50 mg/ml of either cholesteryl ester, cholesterol, phosphatidylcholine, or trioleoylglycerol (Sigma Chemical Co.) were prepared in chloroform. Lipids were used without further purification and stock solutions, stored at −20°C, were replaced at 2-week intervals. [1α,2α(n)-^{3}H]Cholesterol (56 Ci/mmol), cholesteryl [1-^{14}C-oleate] (26 mCi/mmol), tri[1-^{14}C]oleoylglycerol (45 mCi/mmol), and di[1-^{14}C]palmitoylphosphatidylcholine (114 mCi/mmol) were obtained from Amersham. In a typical experiment, 2.37

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; $VLDL_2$ and $VLDL_3$ are subclasses of VLDL with flotation values of S_f 60–100 and S_f 20–60, respectively (9); SDS, sodium dodecyl sulfate; PMCA-oleate, 3-pyrenemethyl-23,24-*dinor*-5-cholen-20-ate-3β-ol oleate; NBD-CL, N-(7-nitrobenz-2-oxa-1,3-diazole)-23,24-*dinor*-5-cholen-22-amine-3β-yl linoleate; apoE, apolipoprotein E isolated from VLDL (2).

[1] Charlton, S. C., A. L. Catapano, P. K. J. Kinnunen, and S. J. Lomasky. 1978. A model triglyceride-rich lipoprotein. *Federation Proc.* **37:** 1320, (Abstract).

mg of cholesteryl ester, 1.41 mg of phosphatidylcholine, 0.52 mg of cholesterol, and 0.7 mg of trioleoylglycerol were mixed and solvent was removed under a strean of N_2. The residue was dried further at room temperature for a minimum of 2 hr by vacuum desiccation. In a typical experiment, 5.0 mg of total lipid was dissolved in 200 μl of 2-propanol previously dried exhaustively over a molecular sieve (Linde Type 3A). The solution was heated to 60°C, and drawn as 66-μl aliquots into a 100-μl Hamilton syringe that was fitted with an unbeveled needle. The syringe temperature was maintained at 55°C with a Hamilton water jacket (Model 875-00). The lipid solution was then injected just below the surface of 1.1 ml of rapidly mixing standard buffer containing 0.15 M NaCl, 25 mM Tris–HCl, and 0.3 mM EDTA, pH 7.4, maintained at 18°C (**Fig. 1**). The 2-propanol was subsequently removed from the solution by centrifugation through 5 ml of buffer-depleted Sephadex G-50. To prepare the buffer-depleted Sephadex column, a plastic 5-ml syringe, fitted with a glass wool plug in the tip to retain the swollen gel, was placed in a 15-ml conical centrifuge tube and centrifuged at low speed to remove the interstitial buffer (10). Each freshly prepared solution, 1.1 ml, was layered on top of the column and was centrifuged through the gel with no dilution. Three separate preparations were combined to give a final volume of 3.3 ml that contained 1.5 mg of lipid/ml, or 2×10^{-4} M, based on phosphatidylcholine content. In one series of experiments, 10^6 cpm 2-[2-^{14}C]propanol (Calif. Bio Nuclear Corp.) was included in the solvent. About 97% of the radioactive solvent was retained in the gel. A second centrifugation through the gel reduced the labeled 2-propanol to 0.7% of the original concentration of solvent. The recoveries of each of the labeled lipids in the cholesteryl ester-rich microemulsions were about 97% following centrifugation through the buffer depleted columns. No selective retention of any of the labels was observed.

In two experiments, the cholesteryl ester-rich microemulsion was prepared by injection into 0.1 M 6-carboxylfluorescein in standard buffer and chromatographed on Sephadex G-50 to remove the free dye. Absorbance of the solution at 492 nm was measured after adding 1 ml of standard buffer containing 0.2% (v/v) Triton X-100 (11). For determination of surface-bound dye, preformed microemulsions were mixed with 0.1 M 6-carboxylfluorescein and chromatographed as before. The difference between total and surface bound fluorophore was considered to be the trapped volume. Egg phosphatidylcholine vesicles were prepared by injection of an ethanolic phospholipid solution (12) into standard buffer containing 6-carboxylfluorescein and chromatographed as done previously.

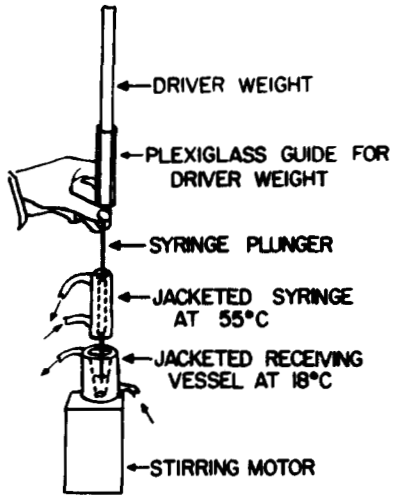

Fig. 1. Apparatus for formation of microemulsions. The 2-propanol solution containing the appropriate lipids was drawn into the 100-μl Hamilton syringe fitted with a Hamilton water jacket maintained at 55°C. The jacketed receiving vessel was positioned so that about 2–3 mm of the syringe needle extended below the surface of the stirred buffer solution at 18°C. The driver weight, a 325-gm brass rod, was placed in the plexiglass guide cylinder to rest on the plunger head. The driver weight and the syringe plunger were released simultaneously to produce the microemulsions.

Column chromatography

Microemulsions were sized by ascending column chromatography on Biogel A-150M in 0.15 M NaCl containing 10 mM Tris–HCl, and 0.3 mM EDTA, pH 7.4. Human $VLDL_2$, $VLDL_3$, and LDL were isolated by rate zonal centrifugation (13, 14) and were a generous gift of Dr. Joseph R. Patsch. The sizes of these lipoproteins were established by laser light scattering (15) and electron microscopy.

Electron microscopy

Samples were diluted as much as 100-fold in 1% potassium phosphotungstate, pH 7.0, containing 200 μg/ml bacitracin to aid spreading. Formvar-coated, carbon-stabilized copper grids were floated for 30 sec on drops of the samples in phosphotungstate, after which excess fluid was removed by filter paper touched to the edge of the grid. The air-dried grids were examined at 60–100 kv in a Phillips 201 electron microscope. The range of diameters was obtained by measurement of 100 particles.

RESULTS

A column chromatography elution profile of a cholesteryl ester-rich microemulsion containing labeled dipalmitoylphosphatidylcholine and cholesteryl oleate is shown

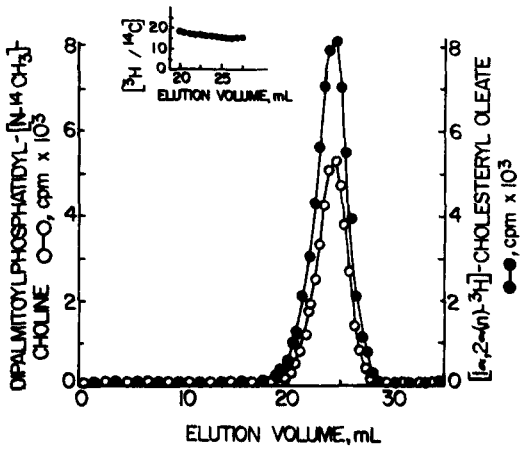

Fig. 2. Gel permeation chromatography of cholesteryl ester-rich microemulsions. A cholesteryl ester-rich microemulsion was prepared containing 0.74 μCi of dipalmitoylphosphatidyl-[N-$^{14}CH_3$]-choline (114 mCi/mmol) and 0.84 μCi of [1α, 2α(n)-^{3}H]-cholesteryl oleate (26 mCi/mmol). A 1.0-ml aliquot was applied to a 1.0 × 40 cm column of Biogel A150-M. The inserts show the ratio of the labeled components across the elution peak. The excluded and included volumes of the column are denoted by V and S, respectively.

in **Fig. 2**. These two major components of the microemulsion coeluted as a single peak which had a fairly constant cholesteryl ester/phospholipid ratio (Fig. 2 insert). For reference, single-walled phosphatidylcholine vesicles appeared as a symmetrical peak in fractions 29–35 of this column. Similar chromatographic profiles were obtained when microemulsions containing radiolabeled phosphatidylcholines and either radiolabeled cholesterol or trioleoylglycerol were analyzed.

The chemical homogeneity and stability of the microemulsions were strictly dependent on the initial composition of the lipid mixtures. When either dipalmitoylphosphatidylcholine, dimyristoylphosphatidylcholine, or 1-stearoyl-2-oleoylphosphatidylcholine were used in amounts equivalent to the total phospholipid content of LDL, chromatographically homogeneous microemulsions were obtained which were optically clear. The average absorbance at 450 nm of six different solutions prepared in a single day was 0.16 ± 0.03. Such microemulsions were stable at room temperature for up to one week. In contrast, microemulsion prepared with dioleoylphosphatidylcholine or egg phosphatidylcholine either commercially obtained or synthesized and purified by high performance liquid chromatography (16), were unstable and became turbid after 3–4 hr or 24 hr, respectively. Cholesterol could be omitted from the lipid mixture without destroying the homogeneity of the microemulsion. By contrast, changes in the composition of the nonpolar neutral core lipids gave substantial alterations in the nature and stability of the dispersions. When the trioleoylglycerol was omitted and the core contained only cholesteryl oleate, a wide range of particles was obtained. About 20% of the cholesteryl ester appeared in the void volume, with another 30% of the material distributed before the main peak at the usual retention volume. Furthermore, this latter microemulsion was extremely unstable. A visible lipid layer frequently appeared at the top of the tube within 15 min.

The stability of the microemulsion also depended on the fatty acyl moiety of the cholesteryl esters. Cholesteryl-oleate and cholesteryl linoleate produced the most stable homogeneous microemulsions. When the lipid mixture contained only saturated cholesteryl esters such as cholesteryl palmitate or cholesteryl myristate, the resulting opaque suspensions could not be chromatographed. Stable microemulsions could also be generated using fluorescent cholesteryl ester analogs. Microemulsions, in which the total content of cholesteryl ester was NBD-linoleate (17), were optically clear and stable. The cholesteryl ester analog, PMCA-oleate (18), could also be used to replace up to 50% of the cholesteryl ester content and produce a stable microemulsions.

Comparison of the elution volume of the cholesteryl ester-rich microemulsions with the elution volumes on A-150M of the VLDL subfractions, $VLDL_2$ and $VLDL_3$, and LDL showed that the microemulsions had a diameter of about 35 nm, a size intermediate between $VLDL_3$ and LDL. Electron microscope examination (**Fig. 3**) showed that the usual preparation of the cholesteryl ester-rich microemulsions contains particles with the diameters varying between 35–70 nm. Quantitative analysis of the particle size (**Fig. 4**) indicate an average diameter of about 45 nm in a typical preparation. Examination of the microemulsions by electron microscopy did not reveal any substantial vesicle contamination but did confirm the gross heterogeneity of particle sizes of microemulsions prepared in the absence of trioleoylglycerol.

To verify the putative structure of the cholesteryl ester-rich microemulsion, the amount of 6-carboxyfluorescein associated with the microemulsion was measured. The trapped dye is a measure of volume contained inside vesicular structures, which are assumed to be impermeable to the dye (11). The difference between total bound and surface-bound 6-carboxyfluorescein was taken as the entrapped volume and was 1.5×10^{-6} ml/μmol phospholipid for the cholesteryl ester-rich microemulsions. The corresponding figure for small single-walled phosphatidylcholine vesicles was approximately 5×10^{-5} ml/μmol phospholipid, in agreement with published results (12). The small amount of trapped dye in the cholesteryl ester-rich microemulsion could be accounted for by a 3% contamination of the microemulsion with single-walled phospholipid vesicles, a heterogeneity that cannot

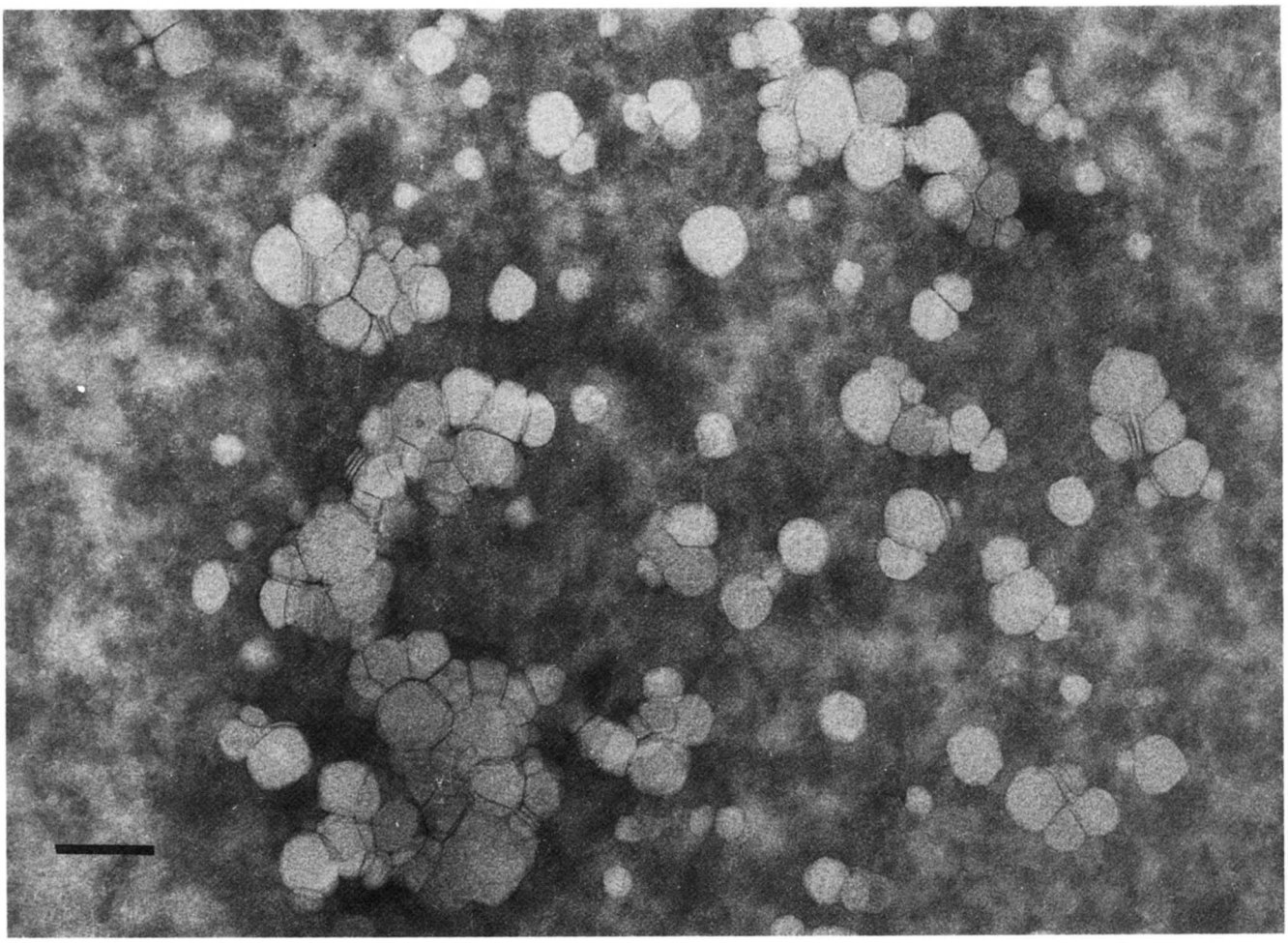

Fig. 3. Electron microscopy of negatively stained cholesteryl ester-rich microemulsions. The particles were negatively contrasted with phosphotungstate as described in Methods. The bar in the lower left corner of the figure equals 100 nm. Magnification 135,000×.

be detected by electron microscopy or column chromatography.

Several potential uses of the cholesteryl ester-rich microemulsion were explored. Since the cholesteryl ester transfer protein of plasma appears to transfer cholesteryl ester between various lipoprotein classes (19), the suitability of the cholesteryl ester-rich microemulsion as a substrate for this reaction was examined. Microemulsions were prepared containing 50% of the cholesteryl ester as PMCA-oleate and incubated with LDL in the presence and absence of lipoprotein deficient plasma at 37°C for 24 hr. LDL was subsequently reisolated by centrifugation and fluorescent spectra obtained. As demonstrated in **Fig. 5**, accumulation of fluorescence in LDL is enhanced markedly by the presence of lipoprotein deficient plasma. In studies from this laboratory described elsewhere, LDL labeled in this manner with nonfluorescent, fluorescent, and isotopically labeled cholesteryl esters are metabolized in a normal fashion when injected into rats and when incubated with human skin fibroblasts (20).

The ability of the cholesteryl ester-rich microemulsion

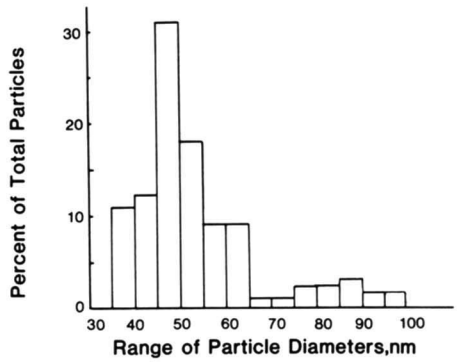

Fig. 4. Range of diameters of negatively stained cholesteryl ester-rich microemulsions. Particles were negatively contrasted with phosphotungstate as described in Materials and Methods. One hundred particles selected at random were measured.

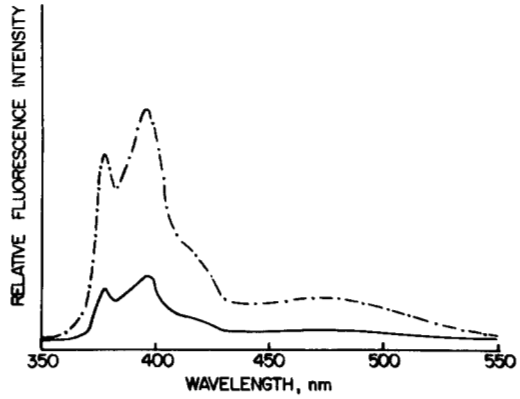

Fig. 5. Incorporation of fluorescent cholesteryl ester into LDL from cholesteryl ester-rich microemulsion. LDL, 2 mg, were incubated with 3.0 ml of cholesteryl ester-rich microemulsion containing PMCA-oleate in the presence or absence of 5 ml of lipoprotein deficient plasma. After 24 hr at 37°C, LDL were reisolated by sequential ultracentrifugation at ρ 1.006 g/ml and ρ 1.063 g/ml. After dialysis, spectra were obtained on an SLM Instrument Model 8000 photon counting spectrofluorimeter (17).

to bind isolated apoliproteins was examined. A microemulsion containing NBD-linoleate was prepared in buffer containing 50 μg/ml ^{125}I-iodo-apoE. The resulting microemulsion was chromatographed on a Sepharose 6B-CL column (**Fig. 6**). About 61% of the radioactivity was associated with the microemulsion in the void volume, where all of the lipid fluorescence appeared. In the absence of the microemulsion, approximately 3.5% of the ^{125}I-iodo-apoE eluted in the void volume fractions while the rest eluted as heterogeneous aggregates throughout the remainder of the column. Moreover, preliminary data indicate that apoE-cholesteryl ester-rich microemulsion complexes are recognized and internalized by hepatic cells during liver perfusion (21).

DISCUSSION

In the present study, we describe the preparation of a stable microemulsion containing the major lipid components of LDL, but without the apoprotein components. The absence of an appreciable trapped volume indicates that the cholesteryl ester-rich dispersion is a microemulsion rather than a dispersion that is vesicular in nature. Examination of the microemulsions by column chromatography indicated an average diameter of 35 nm. By electron microscopy, the mean size of these particles is 45 nm, a value intermediate between those of VLDL and LDL. The microemulsions are relatively homogeneous throughout the peak elution region of the A-150M column with respect to the ratio of cholesteryl ester to phosphatidylcholine and of trioleoylglycerol to cholesterol, phospholipid, and cholesteryl oleate.

It was not possible under our experimental conditions to produce a stable microemulsion containing only phosphatidylcholine, cholesterol, and cholesteryl ester by either sonication or injection. Stable microemulsions are formed ony by injection when triacylglycerol is present. Moreover, the stability of the microemulsion depends on the nature of fatty acyl moiety of the cholesteryl ester. A stable microemulsion could not be produced when saturated cholesteryl esters such as cholesteryl palmitate are substituted for cholesteryl linoleate or cholesteryl oleate. This is in agreement with the recent finding with reconstituted LDL (22) that the cholesteryl esters normally found in LDL, cholesteryl oleate, and cholesteryl linoleate, cannot be replaced entirely by saturated cholesteryl esters.

The method as described in this paper is quite reproducible when sufficient attention is given to several key points. A factor of crucial importance is the temperature of the jacketed syringe. Temperature variations of ±2–3°C give unstable microemulsions. The temperature of the receiving vessel is less critical, with buffer temperatures between 4°C and 25°C providing reasonably stable products. The relation between the thermal properties of the constituent lipids of the microemulsion, the ability to form microemulsions, and the time dependent stability of these microemulsions require additional experiments. The extent to which water has been removed from the 2-propanol is also extremely important. The presence of any significant moisture in the solvent gives unstable microemulsions. Moreover, the length of time over which the lipid stock solutions can be stored is also an important factor. Solutions of trioleoylglycerol

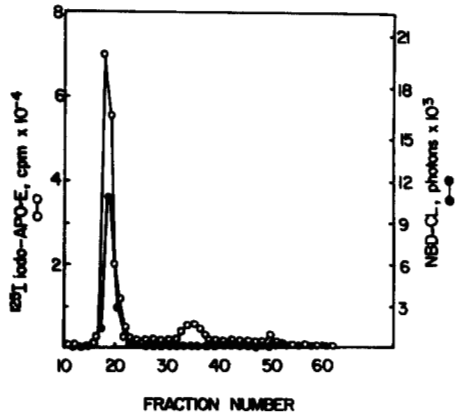

Fig. 6. Binding of I^{125}-iodo-apoE to cholesteryl ester-rich microemulsion. A microemulsion was prepared by injecting the lipid components containing NBD-linoleate into buffer that contained 50 mg/ml ^{125}I-iodo-apoE, with a specific activity of 218 cpm/ng. One ml of the emulsion was chromatographed on a 1.5 × 60 cm Sepharose 6B-CL column. One-ml fractions were collected. Samples were monitored for radioactivity and for fluorescence (photons). Fractions 15–21 represent the void volume of the column.

and phosphatidylcholine are discarded after about 2 weeks.

Evidence for the location of the lipid components in the cholesteryl ester-rich microemulsion is rather indirect. On the basis of the phase diagram for egg yolk phosphatidylcholine, cholesteryl ester, and water (23), a separate phase of cholesteryl ester would be expected to form at the ratios of cholesteryl ester and phosphatidylcholine used in preparation of a cholesteryl ester-rich microemulsion. In addition, it has been demonstrated that trioleoylglycerol and cholesteryl ester are sparingly soluble in phosphatidylcholine (7, 23), and that cholesteryl esters and triacylglycerol are miscible in the amounts used in these experiments (24). Based on the known properties of the various lipid mixtures we have described, a reasonable arrangement of lipids in the cholesteryl ester-rich microemulsion would place the cholesteryl ester and triglyceride in a central core surrounded by the polar lipids in a surrounding monolayer. This is the arrangement of lipid as thought to exist in LDL (25).

The microemulsion described in this report has several potential uses. Since the structure is similar to that of plasma lipoproteins and it associates with apolipoproteins, it will prove to be a useful model for systematic study of the lipid-lipid and lipid-apoprotein interactions of LDL and other cholesteryl ester-rich lipoproteins. In addition, the ability to alter systematically the lipid components of the microemulsion may help define the precise substrate requirements of the various phospholipases and cholesteryl ester hydrolases. Moreover, the cholesteryl ester-rich microemulsion can be used as a substrate for the cholesteryl ester exchange protein of plasma. Exchange of cholesteryl ester between microemulsions and lipoproteins occurs readily, allowing specific labeling of lipoproteins with either radioactive or fluorescent cholesteryl ester analogs, where the extent of lipoprotein labeling is not limited by the solubility of cholesteryl ester in phosphatidylcholine (26). This microemulsion method has been used routinely in our laboratory to incorporate cholesteryl esters into lipoproteins (20, 27).

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