# Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins

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**Abstract** The effect of rat whole blood plasma, serum, serum lipoproteins, and apolipoproteins on the stability of unilamellar liposomes prepared with the French pressure cell was evaluated by measuring the release of entrapped carboxyfluorescein and by electron microscopy. In the absence of serum components, dye escaped very slowly (hours) from egg phosphatidylcholine and phosphatidyl-choline-cholesterol (43 mol % cholesterol) vesicles without apparent change in liposomal structure. This slow release was both temperature- and size-dependent. Serum and some of its constituents induced a far more rapid (seconds) loss of entrapped dye from phosphatidylcholine liposomes, associated with structural changes. For equal masses of protein the order of potency of this induced activity was: free apolipoproteins (apo A-I, apo E) > isolated lipoproteins (HDL and VLDL) > whole serum or whole plasma. Substantial activity was found in three preparations of bovine serum albumin. This activity could be attributed to small and variable amounts of contaminating lipoprotein-like particles and apolipoprotein A-I. Induced release of dye from liposomes by apolipoproteins was usually associated with rapid formation of discs although other structures were sometimes formed. Purified rat apolipoproteins A-I and E appeared to interact identically with liposomes to induce dye release. This effect was progressively impaired for both apoproteins by increasing amounts of cholesterol and was completely inhibited when liposomes contained 37 mol % cholesterol.—**Guo, L. S. S., R. L. Hamilton, J. Goerke, J. N. Weinstein, and R. J. Havel.** Interaction of unilamellar liposomes with serum lipoproteins and apolipoproteins. *J. Lipid Res.* 1980. **21:** 993–1003.

**Supplementary key words** liposomal staiblity · cholesterol · bovine serum albumin · carboxyfluorescein · electron microscopy

Unilamellar liposomes have been proposed as carriers to transport entrapped pharmaceutical agents through the blood to specific sites (1–3). Such liposomes must retain their integrity until they reach the target tissue. In the preceding communication (4), we described a new method of preparing unilamellar liposomes with the French pressure cell and partially characterized them with respect to structure, size, and stability. To evaluate further the stability of these liposomes in the presence of serum, we used a rapid, semiquantitative method that measures the release of a trapped fluorescent dye, carboxyfluorescein (CF) (5).

Two types of unilamellar liposomes might be expected to remain intact in plasma: phosphatidylcholine (PC) liposomes containing cholesterol (6–8) and liposomes prepared with phospholipids that have phase transitions above the physiological temperature (9, 10). In contrast, PC liposomes containing unsaturated acyl chains release trapped materials in the presence of whole blood in vitro (11, 12) or certain serum components: albumin (6, 12, 13) and $\beta$-globulin (14). Krupp, Chobanian, and Brecher (15) first observed that egg PC liposomes were transformed into smaller particles resembling high density lipoproteins (HDL) 5 min after intravenous injection into rats or after 5-min incubation with rat plasma. A similar transformation was produced by mixing liposome preparations with plasma HDL (16–18). Tall and Small (19) reported the appearance of discoidal particles following the incubation of dimyristoyl-phosphatidylcholine (DMPC) liposomes with human HDL. Purified bovine apolipoprotein A-I (apo A-I) also forms "small" complexes with DMPC liposomes containing up to 33 mol % cholesterol (20). However, systematic studies of the stability of unilamellar PC liposomes in serum and the effect of different amounts of cholesterol have not been made.

In this report, we describe a rapid and sensitive method for studying the stability of liposomes in the presence of serum and its components.

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Abbreviations: VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); Apo A-I, Apolipoprotein A-I; Apo E, Apolipoprotein E; CF, carboxyfluorescein; SDS, sodium dodecyl sulfate; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidyl-choline (1,2-dimyristoyl-sn-glycero-3-phosphocholine).
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## MATERIALS AND METHODS

Egg phosphatidylcholine (egg PC) (Sigma, St. Louis, MO) was treated with activated charcoal (Matheson, Coleman and Bell, Norwood, OH) suspended in absolute ethanol to remove colored contaminants. The purity of the egg PC was evaluated by thin-layer chromatography (4). Cholesterol (Nutritional Biochemicals, Cleveland, OH) was recrystallized three times from methanol, and 4(5)-carboxyfluorescein[2] (CF) (Eastman, Rochester, NY) was used as received or further purified as described (5). No differences in rate of slow release (described in a later section) were observed with the two dye preparations. Therefore, CF was ordinarily used as received. Bovine serum albumin was obtained from three sources (Sigma, No. A-4378, crystallized-lyophilized ("I"); Sigma, No. A-4503, fraction V ("II"); and Pentex, Kankakee, IL, No. 82-002, fraction V, fatty acid free ("III")). Fetal calf serum was obtained locally (Univ. of California, San Francisco, lot no. A-225).

### Preparation of liposomes

Ethanol solutions of egg PC with and without cholesterol were evaporated to dryness in a rotary evaporator and dispersed in either 0.2 M aqueous CF, pH 7.7 or a "standard buffer" containing 0.15 M NaCl, 0.02 M Tris, pH 7.7. Liposomes were then prepared from these dispersions (4–10 mg/ml) with a French pressure cell (American Instrument Co., Silver Spring, MD) at 20,000 PSI at room temperature (4). Liposomes containing CF within the trapped volume were separated from untrapped dye at 4°C on a column of Sephadex G-50 (2 × 10 cm) equilibrated with the standard buffer and were used the same day. In some experiments, liposomes were purified by chromatography on a 2% agarose column (1.2 × 95 cm) or by ultracentrifugation at 100,000 $g$ for 30 min at 4°C to remove small amounts of multilamellar liposomes.

### Assay of dye release from liposomes

The fluorescence of CF is largely self-quenched at concentrations above about 0.01 M, whereas in dilute solution the fluorescence is proportional to concentration (5). The concentration of dye within the liposomes was approximately 0.2 M so that an increase in emission readings indicated release of trapped dye. Liposomes containing CF were monitored to determine their integrity prior to use.

Release of dye was measured with a spectrophotofluorometer (Model SPF-125, American Instrument Co.) at 460 nm excitation and 520 nm emission wavelengths. Ten $\mu l$ of liposomes containing CF (40–50 $\mu g$ lipid) was rapidly mixed with 1 ml of buffer, serum, or serum protein solutions at 37°C, and the intensity of fluorescence was recorded continuously. At the end of the experiment, 0.1 ml of Triton solution (20%) was added to the mixture to release dye remaining within the liposomes. The % of dye release was calculated with the following formula:

$$100\ (F - F_0)/(F_T \times 1.1)$$

where $F_0$ is the fluorescence intensity of 1 ml of buffer plus 10 $\mu l$ dye-containing liposomes at time 0 (about 4–8% of the total fluorescence was usually present in $F_0$); F is the fluorescence intensity of 1 ml of buffer or serum proteins plus 10 $\mu l$ dye-containing liposomes at time T; and $F_T$ is the fluorescence intensity of the above mixtures after addition of 0.1 ml of Triton solution.

Serum or serum proteins cause substantial quenching of dye fluoresence that is increased in the presence of Triton. Therefore, post-Triton quenching of each serum protein was determined in the presence of free-dye, and the corresponding $F_T$ value was corrected for quenching attributed to Triton. These quenching effects were not observed when serum proteins were diluted to about 1 mg/ml or less.

Two different types of release were observed: 1) a slow temperature-dependent release was found to occur over a period of hours; 2) a very rapid release was induced within seconds to a few minutes after addition of whole serum or some of its constituents.

### Preparation of blood serum and serum protein fractions

Serum and plasma (to which 0.1% EDTA was added) were obtained from fasted normal adult humans or male adult rats of the Sprague-Dawley strain. Rat plasma lipoproteins (very low density lipoproteins (VLDL), d < 1.006 g/ml, and high density lipoproteins (HDL), 1.075 < d < 1.21 g/ml), were isolated by sequential ultracentrifugation (21). Apolipoproteins were prepared by delipidation as described (22). For apo VLDL, the tetramethylurea-soluble fraction (23) was used. Apolipoprotein E (Apo E) and apolipoprotein A-I (apo A-I) were purified from apo HDL on a column of Sephadex G-200 (1.2 × 300 cm) equilibrated with 4 M guanidine–HCl, 0.01 M Tris, pH 8.2. To remove contaminating lipoproteins from commercial bovine serum albumins, a 5% solution was adjusted to a density of 1.25 g/ml with KBr and separated by ultracentrifugation at 100,000 $g$ for 24 hr. Rabbit IgG was prepared as described by Williams and Chase (24). Serum protein fractions were dialyzed against standard buffer.

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[2] This compound was previously distributed as 6-carboxyfluorescein, but is now thought to consist of both the 4- and 5-carboxyfluorescein isomers.
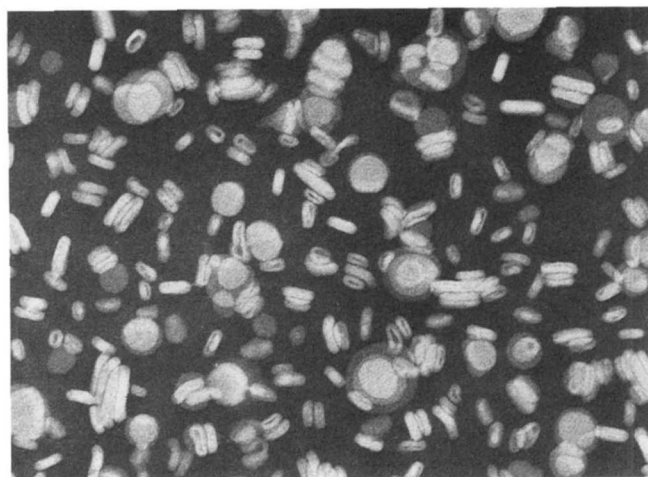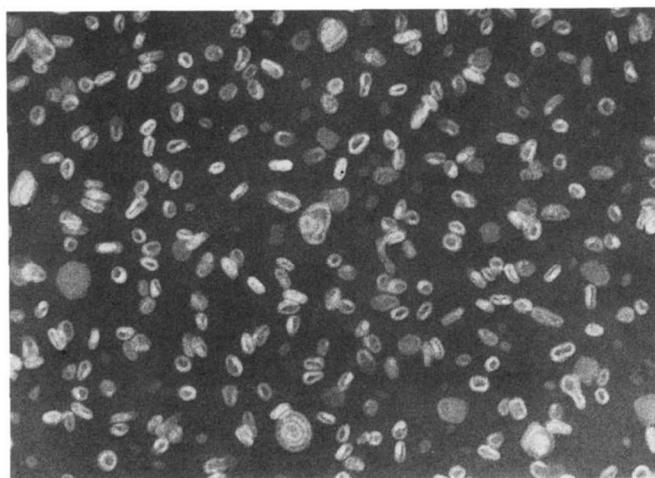
**Fig. 1.** (×120,000) Negatively stained preparations of liposomes prepared in the presence of CF (0.2 M, pH 7.7). Left: PC liposomes. Right: PC-cholesterol liposomes, 43 mol % cholesterol.

## Incubation of serum protein fractions with phosphatidylcholine liposomes

Protein solutions of varying concentrations were incubated with liposomes at 37°C for 3 hr. The lipid or lipid-protein complexes were separated by ultracentrifugation after adjusting the density to 1.21 g/ml with KBr. All isolated fractions were recentrifuged once at the same density.

## Chemical analysis

Electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) was performed as described (22). Protein was determined by a modification of the procedure of Lowry et al (25) with bovine serum albumin as standard. Cholesterol was determined by a ferric chloride method (26). Lipid phosphorus was measured according to Bartlett (27).

## Electron microscopy

Negatively stained preparations of liposomes or lipid-protein complexes were prepared and sized as described (4, 28). They were examined and photographed at 60,000 diameters and 80 kV in a Siemens 101 electron microscope (Siemens Corp., Medical/ Industrial Groups, Iseline, NJ).

## RESULTS

## Electron microscopy of liposomes containing carboxyfluorescein

In negative stains, both PC and PC-cholesterol liposomes prepared in the presence of CF (**Fig. 1**) appeared to be primarily single bilayer vesicles which were larger when cholesterol (43 mol %) was incor-

porated. The elution profile of the dye-containing liposomes from a 2% agarose column (**Fig. 2**) was similar to that of liposomes formed in the absence of dye (4). However, the liposomes prepared in the dye solution appeared to have a slightly larger mean particle size (248 Å vs 204 Å) as determined from electron microscopic images of negatively stained samples from the ascending slope, peak, and descending slope of the elution-profile. The values given represent weighted averages of measurements from Fig. 2 of the present paper and from Fig. 3 of the preceding paper (4).

## Stability of liposomes containing dye

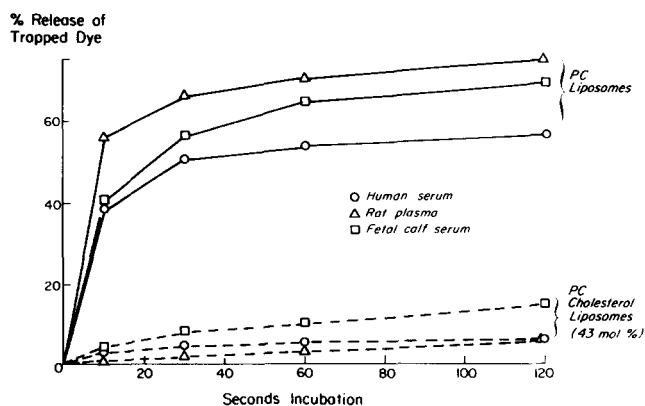PC-cholesterol liposomes released no detectable dye during 3-hr storage in buffer at 4°C. A small
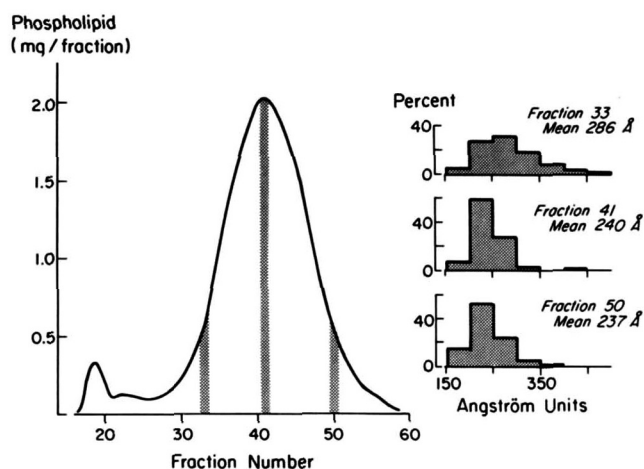


**Fig. 2.** Left: Elution (from a 2% agarose column, 1.2 × 95 cm) of PC liposomes containing CF. Right: Mean particle diameter and distribution of diameters of unilamellar liposomes of different elution volumes. Particle diameters were measured from negatively stained images by described methods (4, 45).

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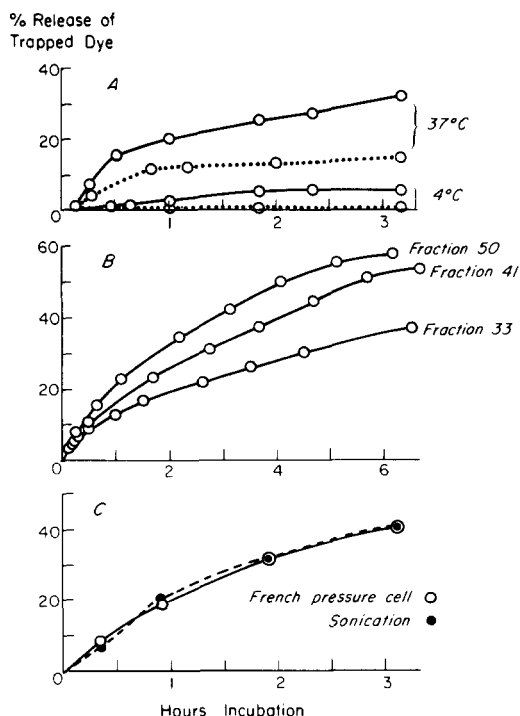
**Fig. 3.** Release of CF from liposomes. A. Release of dye from PC liposomes (solid line) and, PC-cholesterol liposomes (43 mol % cholesterol, dotted line) at various temperatures. B. Release of dye from fractions of PC liposomes, eluted from a 2% agarose column at 37°C, as in Fig. 2. C. Release of dye from PC liposomes prepared by French pressure cell (○) or sonication (●) at 37°C and purified by chromatography on 2% agarose gel. Liposomes were from the fraction corresponding to fraction 41 in Fig. 2.

amount of dye (5%) was progressively released during the first 2 hr from liposomes lacking cholesterol (**Fig. 3A**). Prolonged storage of both types of liposomes at 4°C for up to 20 hr did not cause appreciable further loss of dye (not shown). A considerable amount of dye was released (Fig. 3A) at 37°C over a period of several hours. This slow release of dye



**Fig. 4.** Induced release of CF from liposome by whole serum or plasma at 37°C.

**TABLE 1.** Induced release of CF from liposomes: relative activity of various serum proteins

| Protein | | % Release of Trapped Dye in One Minute | |
| | | PC Liposomes | PC-Cholesterol Liposomes |
| --- | --- | --- | --- |
| | (mg/ml) | | (43 mol % cholesterol) |
| Rat apo HDL | 0.5 | 78[a] | 31[a] |
| Rat apo HDL | 0.05 | 51 | 0 |
| Rat apo HDL | 0.005 | 21 | 0 |
| Rat apo VLDL | 0.5 | 77 | 31 |
| Rat HDL | 1.0 | 40 | 0 |
| Rat VLDL | 1.0 | 25 | 0 |
| Human serum | 1.0 | 13 | 0 |
| Fetal calf serum | 1.0 | 7.4 | 0 |
| Rat plasma | 1.0 | 3.4 | 0 |
| Bovine serum albumin[b] | 1.0 | 3.1 | 0 |
| Rabbit IgG | 1.0 | 0 | 0 |

[a] Numbers are means of duplicate assays.
[b] Sigma, fraction V.

was nonlinear with time and was considerably greater for PC than PC-cholesterol liposomes (32% vs 14% after 3 hr of incubation). Loss of dye at 25°C was slower than at 37°C (not shown). To determine if the nonlinear slow release of dye was a function of the size of unilamellar liposomes, we measured the rate of dye release from different liposome fractions eluted from a 2% agarose column (Fig. 2). Release at 37°C was faster from smaller liposomes (Fig. 3B). No difference in the rate of release was observed for liposomes of the same elution fraction (i.e., same size) prepared with the French pressure cell or by sonication (Fig. 3C).

## Instability of liposomes induced by serum constituents

Serum or plasma from humans, rats, or fetal calves induced very rapid loss of entrapped CF at 37°C from egg PC liposomes (**Fig. 4**). Dye release was nonlinear with time, when plotted on Cartesian or semilogarithmic coordinates, with a substantial rapid component occurring during the first 30 sec of incubation. Inclusion of 43 mol % cholesterol into the liposomes markedly reduced the rate of release of dye in all instances (Fig. 4).

When serum or plasma was diluted to the same protein concentration (1 mg/ml) used in testing serum protein fractions (**Table 1**), dye-releasing activity was in the following order: apolipoproteins > lipoproteins > serum, plasma, or albumin. Only the apolipoproteins produced loss of trapped CF (31% in 1 min) from egg PC-cholesterol liposomes (43 mol % cholesterol). Rabbit gamma globulin produced no loss of trapped dye.
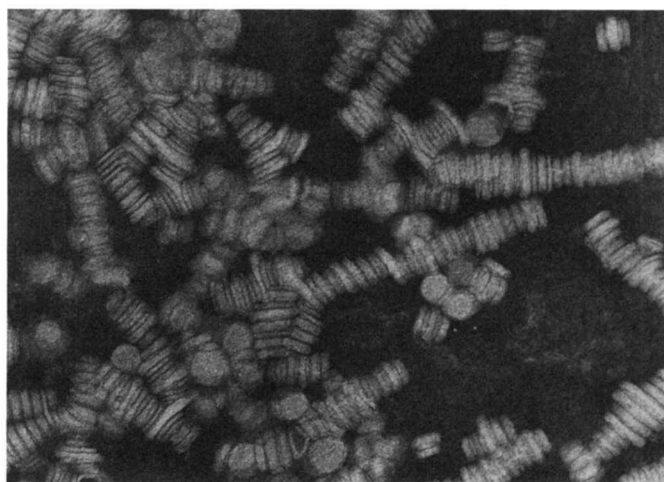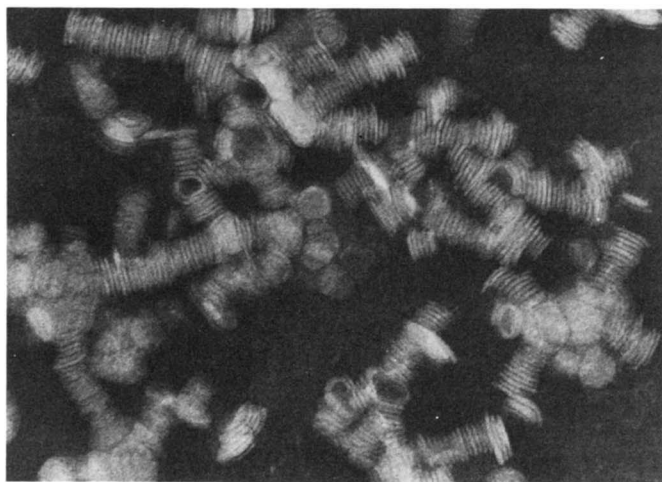
**Fig. 5.** (×120,000) Images obtained by negative staining of mixtures of liposomes and rat apo HDL (lipid–protein 2:1 w/w) incubated at 37°C for 5 min. Left: PC liposomes appear almost completely transformed into discoidal particles with an edge thickness of about 35 Å, appropriate for a single bilayer. (The stacking in rouleaux is an artifact of negative staining that occurs more in the presence of apoproteins). Right: PC-cholesterol liposomes (43 mol % cholesterol) appear to remain intact as shown by the thickness of the edges of individual particles which slightly exceed 100 Å. A few particles in the rouleaux, however, appear to be discs consistent with the data in Fig. 4.

## Structural changes of liposomes associated with induced dye release

After incubating PC liposomes with rat apo HDL (phospholipid–protein 2:1, w:w) at 37°C for 5 min, we found that they had been transformed into discs with few or no intact liposomes remaining (**Fig. 5, left**).[3] In contrast, a mixture of PC-cholesterol liposomes (43 mol %) and rat apo HDL appeared to contain mainly intact liposomes with few discs (Fig. 5, right). The same results were obtained in each case with longer incubations up to 3 hr. The electron microscopic appearance of the mixtures of phosphatidylcholine liposomes with rat apo HDL (**Fig. 6B**) and rat apo VLDL (Fig. 6C) differed. The former produced mainly discs with few or no intact liposomes, whereas the latter produced a heterogeneous mixture of discs, larger multilamellar structures, and very small particles. No detectable changes of either structure or size were observed when egg PC liposomes were incubated alone at 37°C for 3 hr (Fig. 6A).

## Identification of major apolipoproteins that change liposomal structure

To determine which apoproteins of HDL and VLDL associate with liposomal lipids, lipid-protein

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[3] To distinguish discs and intact liposomes in an electron microscopic image of negatively stained preparations, the particles must form stacks in rouleaux so that the edge thickness of individual particles can be measured. The thickness of the edge of intact liposomes is about 100 Å because it represents the sum of two bilayer thicknesses plus a small and variable internal volume (29). Single bilayers of PC alone or with cholesterol are about 35–45 Å thick (30, 31). The interpretation of the images obtained by negative staining has been confirmed by thin-sectioning techniques (4, 29).

complexes were separated after 3 hr of incubation (37°C) from the remaining apoproteins by ultracentrifugation at a density of 1.21 g/ml. Apo A-I was the major component in SDS polyacrylamide gel electrophoretograms of these isolated lipid-protein complexes (**Fig. 7**) when liposomes were incubated with rat apo HDL. Only small amounts of the other apoprotein components of HDL (apo A-IV, apo E, apo C) were associated with lipid. When rat apo VLDL was in the incubation mixture, apo E was the major apoprotein, and apo C was a minor component of the isolated complexes (Fig. 7).

## Effect of cholesterol on stability of liposomes exposed to pure apolipoproteins

Purified rat apo E and A-I were incubated with PC liposomes containing different amounts of cholesterol (**Table 2**). No differences in the rate or amount of dye released by these apoproteins were detected. As the proportion of cholesterol in the liposomes was increased, less dye was released by each apolipoprotein until the process was completely inhibited in liposomes containing 37 mol % cholesterol. Lipid-protein complexes prepared from PC liposomes with either apo E or A-I appeared to be mainly discoidal particles similar to those seen with apo HDL (Fig. 5, left) whereas liposomes containing cholesterol appear unaltered (Fig. 5, right).

## Identification of apo A-I as a lipid-binding contaminant of bovine serum albumins

Several reports describe interactions between bovine serum albumin and liposomes (6, 12, 13, 32).
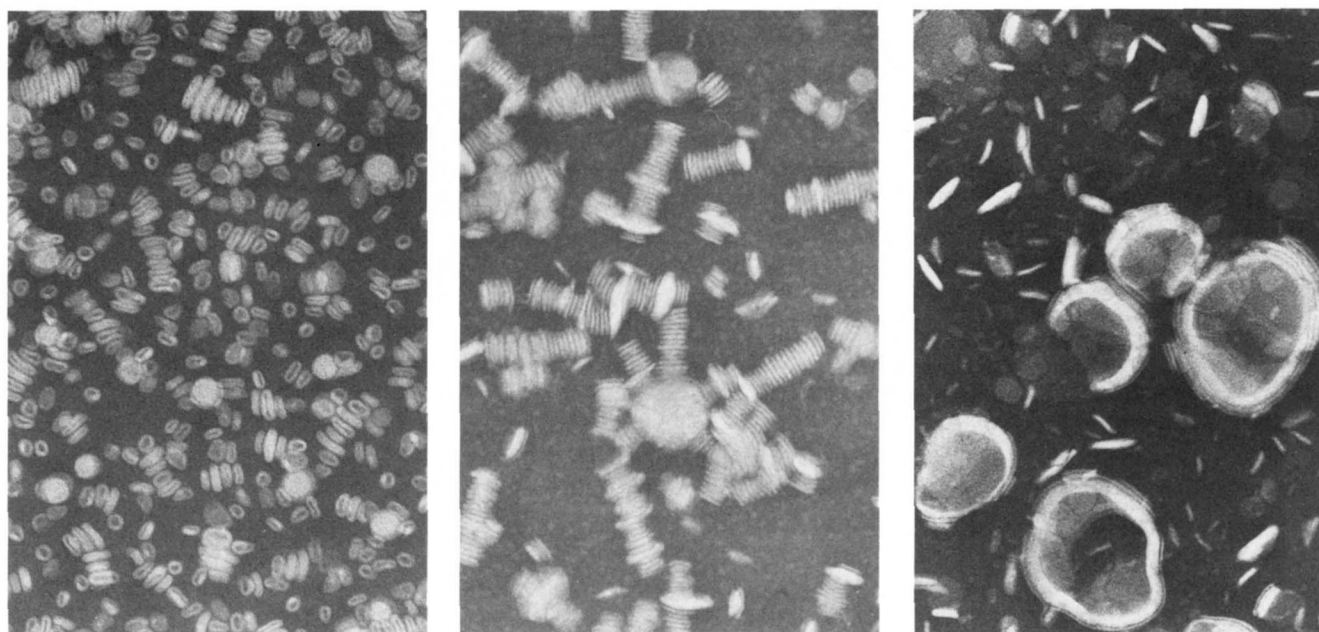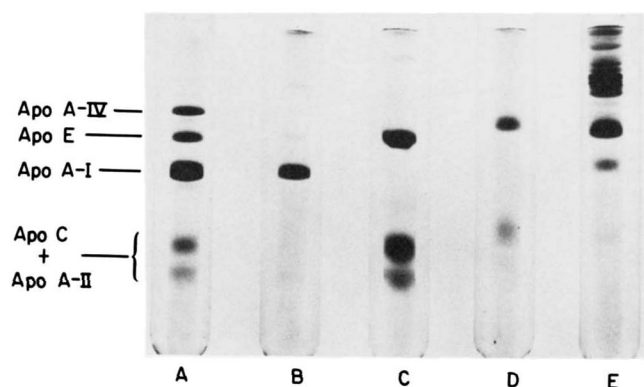
**Fig. 6.** (×120,000) Images obtained by negative staining after PC liposomes were incubated at 37°C for 3 hr with either (A) buffer (0.15 M NaCl, 0.02 M Tris, pH 7.7). (B) rat apo HDL, or (C) rat apo VLDL (lipid–protein 2:1 w/w). Left: (A) Liposomes appear unaltered as shown by the edge thickness (>100 Å). Middle: (B) Discoidal structures predominate and appear the same after 3 hr incubation as after 5 min (Fig. 5). Right: (C) Multilamellar particles and discoidal structures predominate although there appear to be some intact liposomes together with smaller particles in this heterogeneous mixture.

To determine if contaminating lipoproteins or apolipoproteins were responsible for the rapid dye release induced by albumin, we prepared three commercial bovine serum albumins at physiological con-



**Fig. 7.** Identification of apolipoproteins from isolated lipid–protein complexes in SDS polyacrylamide gel electrophoretograms. PC liposomes were incubated with apoproteins (lipid–protein 2:1 w/w) for 3 hr at 37°C except for E in which the d > 1.21 g/ml fraction of rat serum was incubated with liposomes at a weight ratio of lipid to protein of 1:50. All lipid–protein complexes were reisolated by ultracentrifugation at d = 1.21 g/ml. A and C identify major apolipoproteins of rat HDL and VLDL, respectively (25 μg protein). B. Lipid-protein complexes formed by rat apo HDL and liposomes (10 μg protein). D. Lipid-protein complexes formed by rat apo VLDL and liposomes (10 μg protein). E. Lipid-protein complexes formed by apoproteins contained in d > 1.21 g/ml infranatant of rat serum (25 μg protein).

centration (50 mg/ml) and separated them into "heavy" and "light" density fractions (greater or less than 1.25 g/ml) by ultracentrifugation at 100,000 g for 24 hr. These three albumin preparations induced rapid dye release (26–92% in 1 min) from PC liposomes (**Table 3**). Moreover, all samples of both heavy and light ultracentrifugal fractions produced substantial and rapid release of trapped dye (Table 3). Each light fraction appeared by electron microscopy to contain different amounts of lipoprotein-like material that corresponded to measurable amounts of lipid phosphorus (Table 3); thus, the albumin (Pentex) with the most phospholipid also contained much more particulate material (**Fig. 8**). Bands corresponding in mobility to apo A-I and apo C of human HDL and to albumin (**Fig. 9**) were seen in SDS polyacrylamide gel electrophoretograms of the proteins of these light fractions.

The heavy fractions (which should be free of lipoprotein-like material) had even greater dye-releasing activity than the corresponding light and parent fractions (Table 3). To remove the active components from heavy (d > 1.25 g/ml) fractions (Table 3) and to identify them, we incubated egg PC liposomes with each of the three heavy albumin fractions (phospholipid–protein 1:50, w:w) at 37°C for 3 hr. The lipid and the lipid-protein complexes thus formed were then separated from albumin by ultracentrifu-

TABLE 2. Induced release of CF from liposomes containing varying amounts of cholesterol by rat apo E and apoA-I

| Time | Liposomes (Mol % Cholesterol)[a] | | | | | | | | | |
| | 0 | | 8.3 | | 16.6 | | 28.0 | | 37.4 | |
| | Apo E | Apo A-I | Apo E | Apo A-I | Apo E | Apo A-I | Apo E | Apo A-I | Apo E | Apo A-I |
| (sec) | (% release of trapped dye) | | | | | | | | | |
| 10 | 13.7[b] | 17.6 | 4.2 | 4.0 | 5.4 | 8.2 | 2.2 | 0 | 0 | 0 |
| 30 | 22.8 | 24.5 | 12.0 | 12.4 | 11.1 | 14.4 | 3.5 | 1.7 | 0 | 0 |
| 60 | 28.1 | 29.1 | 17.3 | 16.8 | 15.5 | 18.7 | 4.4 | 2.6 | 0 | 0 |
| 120 | 33.4 | 34.5 | 23.7 | 22.3 | 19.0 | 23.7 | 4.8 | 4.4 | 0 | 0 |

[a] Contents of cholesterol were determined on unilamellar liposomes purified by chromatography on Sephadex G-50 and then centrifuged at 100,000 g to remove small amounts of multilamellar particles.

[b] Numbers are means of duplicate assays.

Experiments were carried out by adding 15 $\mu$g of liposome-PC (10 $\mu$l) to 1 ml of standard buffer containing 10 $\mu$g of apolipoproteins at 37°C.

gation (d = 1.21 g/ml) at 100,000 g for 24 hr. Trace amounts of lipid phosphorus (1–3 $\mu$g/100 mg albumin) remained in the treated albumin fractions (d > 1.21 g/ml). Experiments with CF-containing liposomes (**Table 4**) showed that exposure to liposomes completely removed the release-promoting activity from all three albumin preparations. Small amounts of protein (0.03–0.1% of the total protein) were associated with the lipid-protein complexes (d < 1.21 g/ml). SDS polyacrylamide gel electrophoretograms (Fig. 9) of these complexes contained a protein band corresponding in mobility to human apo A-I in addition to albumin. These lipid-protein complexes were delipidated, and the proteins were found to retain dye-releasing activity (not shown).

### Removal of apolipoproteins from lipoprotein-depleted serum (d > 1.25 g/ml)

Rat serum, from which the major lipoprotein classes have been removed by ultracentrifugation at density of 1.21 g/ml, is known to contain small amounts of apolipoproteins (33). Two such preparations of lipoprotein-depleted rat serum (50 mg protein/ml) caused approximately 43% release of trapped dye in 2 min from liposomes (not shown). This activity was abolished by incubation with PC liposomes. Polyacrylamide gel electrophoretograms of isolated lipid-protein complexes separated by ultracentrifugal flotation at a density of 1.21 g/ml, (Fig. 7E) contained bands corresponding to rat apo E and A-1.

### DISCUSSION

These studies show that unilamellar liposomes prepared with the French pressure cell, with or without entrapped CF, are similar to the commonly used sonicated liposome preparations. Release of trapped CF provides a rapid and sensitive method to assay the structural integrity of liposomes in vitro. Release of CF from liposomes in the absence of serum or serum proteins occurred slowly over a period of hours and was temperature-dependent. No difference was found in the rate of release of dye from liposomes of the same size (mean 240 Å) prepared by the French

TABLE 3. Induced release of CF from liposomes by commercial bovine albumins and their ultracentrifugal fractions[a]

| | % Release of Trapped Dye in One Minute |
| --- | --- |
| Bovine albumin I (Sigma, crystallized-lyophilized) | |
| Original | 53[b] |
| d < 1.25 g/ml | 39 |
| d > 1.25 g/ml | 67 |
| Bovine albumin II (Sigma, fraction V) | |
| Original | 26 |
| d < 1.25 g/ml | 13 |
| d > 1.25 g/ml | 42 |
| Bovine albumin III (Pentex, fraction V) | |
| Original | 92 |
| d < 1.25 g/ml | 81 |
| d > 1.25 g/ml | 90 |

[a] Albumin solutions (50 mg/ml) were separated by ultracentrifugation into two density fractions at a density of 1.25 g/ml. The light fractions usually contained less than 0.2% of the original proteins. Phospholipid contents were approximately 0, 38, and 280 $\mu$g/g of the total protein for Albumin I, II, and III, respectively. Experiments were carried out by adding 10 $\mu$l of liposomes (40–50 $\mu$g phospholipids) to 1 ml albumin solutions containing about 50 mg protein in original and d > 1.25 g/ml fractions and about 0.1 mg protein in d < 1.25 g/ml fractions.

[b] Numbers are means of duplicate assays.
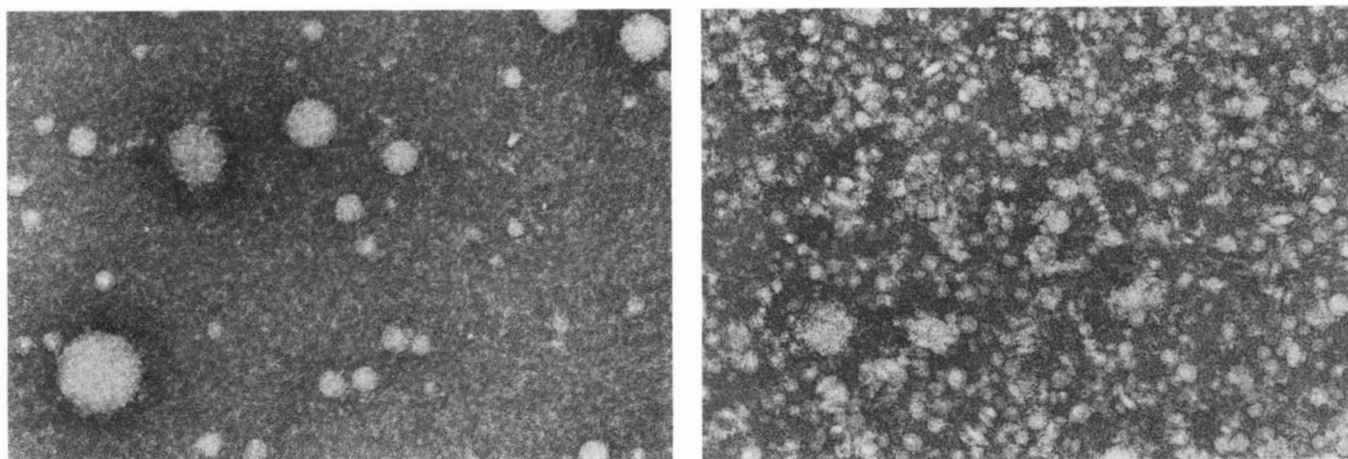
**Fig. 8.** (×180,000) Negatively stained preparations of d < 1.25 g/ml fractions from bovine albumins II (Sigma, fraction V, left) and III (Pentex, fraction V, right). Many particles appear similar in size to HDL (100 Å diameter) in the Pentex light fraction (right) whereas they are far more heterogeneous ranging from about 100 Å to 1,200 Å diameter in the Sigma light fraction (left).
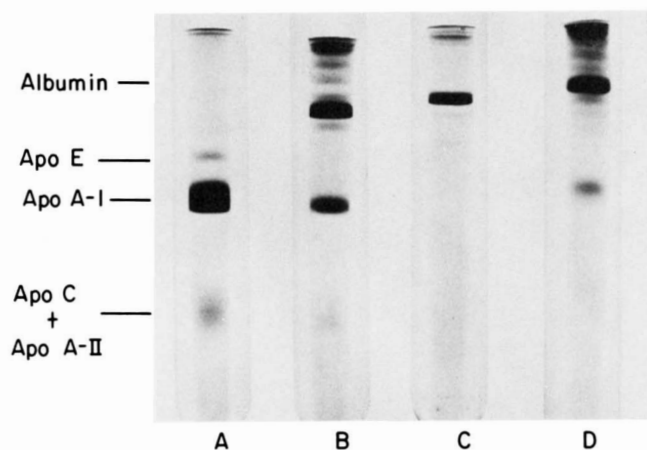
pressure cell or by sonication. This finding differs from that of Barenholz, Amselem, and Lichtenberg (34) who reported a 14-fold greater release of dye from sonicated liposomes. Because the structure of liposomes was apparently unaltered after incubations without protein for 3 hr at 37°C that released a substantial fraction of trapped dye (Fig. 6A), this slow loss presumably occurred by diffusion across the membrane as with other substances of low molecular weight (6, 35). This release evidently was faster from smaller liposomes.

Exposure of liposomes to serum or serum components induced a much faster release of trapped



**Fig. 9.** Identification of Apo A-I in commercial bovine albumin in SDS gel electrophoretograms. (25 μg protein was applied in each case). A. Human apo HDL. B. d < 1.25 gm/ml fraction from bovine albumin III (Pentex, fraction V). C. d > 1.25 gm/ml fraction from bovine albumin III (Pentex, fraction V). D. Lipid-protein complexes obtained by incubation of liposomes with d > 1.25 g/ml fraction (lipid–protein 1:50 w/w).

dye, with more than 50% released within 1 min at 37°C. Previous studies with time-consuming dialysis techniques also demonstrated the release of markers, differing in molecular weight, from sonicated liposomes incubated with whole blood or serum albumin (9, 12). However, the rate of release in these reports appeared to be much slower than the protein-induced release observed in our experiments and could not be distinguished from the slow release owing to diffusion across the membrane.

Our combined electron microscopic and biochemical data show that the major structural conversion of liposomes to discoidal particles occurs in less than 5 min after mixing unilamellar vesicles and rat apolipoproteins A-I and E at 37°C. Apo A-I from other species reportedly forms discoidal complexes from sonicated liposomes of different phospholipids (36–38). Thus, the general process of free apoprotein interaction with unilamellar liposomes to form discoidal particles with release of the entrapped contents evidently is similar for vesicles prepared by the French pressure cell and by sonication.

We found serum apolipoproteins to be the most potent liposome-disrupting agents of serum. Free apolipoproteins may exist in native serum, but the amounts must be too small to account for the observed serum activity. However, Nichols et al. (18) have reported that incubation of human HDL with sonicated unilamellar liposomes of DMPC results in uptake of DMPC by the HDL and dissociation of lipid-free apo A-I which forms discs with remaining vesicles. In similar studies using human HDL and sonicated egg PC vesicles containing trace cholesteryl esters, Chobanian, Tall, and Brecher (17) found that

interactions were dependent on time, temperature, and concentration of reactants, and concluded that they resulted from collisions between vesicles and HDL (17). Apo A-I is loosely associated with human HDL and may be dissociated in vitro under several mild conditions (19, 39). Although our studies are based largely on rat apo A-I and apo E, it is probable that the induced release of dye and disc formation from our liposomes were mediated by similar processes.

Reports of albumin binding to liposomes (6, 12, 13) may be explained by the presence of small amounts of contaminating lipoproteins or apolipoproteins. Previously, we reported in a review that some commercial preparations of bovine serum albumin contain substantial amounts of HDL that appear as lamellar particles by electron microscopy (29). We found that different commercial preparations of bovine albumins contained variable amounts of contaminants that induce the release of CF from liposomes. We also showed that contaminating lipoprotein-like particles can be eliminated by ultracentrifugation and that the remaining free apoproteins can be removed by absorption with liposomes followed by flotation of the lipid-apoprotein complexes. The observation that $d > 1.25$ g/ml fractions of bovine serum albumin were more potent than the parent albumin in inducing dye release may be explained by the presence of free apoprotein in these fractions. The $d > 1.25$ g/ml fractions from serum known to contain free apolipoproteins (33) also induced dye release, and in all experiments we found that lipid-free apolipoproteins were the most potent dye-releasing agents tested.

The major free apoprotein in bovine albumin that caused the release of CF from liposomes was probably apo A-I, although our experiments do not exclude the possibility that other proteins or lipoproteins are involved as well. Fainaru and Deckelbaum (40) quantified bovine A-I in commercial preparations by radioimmunoassay and found that the amount of this contaminant varied greatly among different preparations.

Inclusion of cholesterol in PC liposomes (43 mol %), remarkedly reduced disc formation because intact liposomes were the predominant species remaining when cholesterol-containing liposomes were incubated with apo HDL (Fig. 5). However, a few discoidal particles were also found, suggesting a heterogeneous distribution of cholesterol between or within liposomes as reported in the accompanying paper (4), with cholesterol-poor liposomes or regions being more easily disrupted by the apoproteins. This may explain in part why apo HDL and apo VLDL

TABLE 4.    Removal of lipid-binding proteins from commercial bovine albumins by exposure to liposomes

| | % Release of Trapped Dye Seconds | | | |
|---|---|---|---|---|
| | 10 | 30 | 60 | 120 |
| Albumin I[a] (Sigma, crystallized-lyophilized) | | | | |
| Control | 4.7[c] | 11.0 | 15.6 | 23.2 |
| Incubated[b] | 3.4 | 3.4 | 3.4 | 3.4 |
| Albumin II (Sigma, fraction V) | | | | |
| Control | 3.1 | 7.6 | 9.7 | 12.6 |
| Incubated | 2.2 | 2.2 | 2.2 | 2.2 |
| Albumin III (Pentex, fraction V) | | | | |
| Control | 30.7 | 47.0 | 53.6 | 55.5 |
| Incubated | 4.5 | 4.5 | 4.5 | 4.5 |

[a] All albumins were first ultracentrifuged at a density of 1.25 g/ml for 24 hr at 100,000 g to remove lipoprotein particles. The heavy fractions (d > 1.25 g/ml) were dialyzed thoroughly against standard buffer and used for the experiment.
[b] Liposomes were mixed with the control albumin preparation (lipid–protein 1:50 w/w) and incubated at 37°C for 3 hr. Lipid–protein complexes were then removed by ultracentrifugation at d 1.21 gm/ml. The resulting d > 1.21 g/ml fractions were tested after dialysis. All tested albumin solutions were 30 mg protein/ml.
[c] Numbers are means of duplicate assays.

released some dye from cholesterol-containing liposomes when the concentration of these free apolipoproteins was increased many fold (see Table 1).

By using unilamellar liposomes containing increasing amounts of cholesterol, we found two major apolipoproteins of rat serum, apo E and apo A-I, to interact similarly with liposomes to induce release of dye. As the molar ratio of cholesterol was increased, less dye was released by these apoproteins until dye release was completely inhibited at 37 mol % cholesterol. This inhibition of dye release was consistent with electron microscopic images indicating that the conversion of liposome to disc by apoprotein was prevented. Our findings confirm and extend the reports of others who showed that formation of complexes between human apo A-I and multilamellar DMPC (41, 42), PC from human HDL (41), or sphingomyelin (41) was progressively inhibited as the cholesterol was increased to 33 mol % (41, 42). With mixtures of bovine apo A-I and unilamellar DMPC-cholesterol liposomes prepared by sonication, Jonas and Krajnovich (20) also found that formation of a "small" complex was completely inhibited when liposomes contained 37 mol % cholesterol. Because we found that cholesterol also prevented disruption of liposomes by whole serum from several species, cholesterol appears to have a general effect of inhibiting binding of certain mammalian apolipoproteins to liposomes with consequent disruption to form discs. The present experiments

suggest that the more stable cholesterol-containing liposomes are remarkably similar to the abnormal lipoprotein commonly called LP-X, found in the plasma of humans or rats with cholestasis. LP-X is a bilayer vesicle composed of equimolar phospholipids and unesterified cholesterol with very small amounts of bound apolipoprotein (43, 44). Its stability in plasma evidently is explained by its lipid composition.▣

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