Reassembly of Lipid-Protein Complexes of Pulmonary Surfactant

PROPOSED MECHANISM OF INTERACTION*

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We studied the interaction at 37°C between a major apolipoprotein of pulmonary surfactant and 11 mixtures of lipids. The experiments were carried out in the presence of either 3 mM Ca⁺⁺ or 10 mM EDTA. The amount of apolipoprotein associated with lipid was independent of Ca⁺⁺. However, the binding was sensitive to the percentage of gel-state lipid in the vesicles, and the amount of apolipoprotein in the reconstituted lipoprotein complex decreased as the percentage of fully saturated phospholipid was reduced. Maximum association of the apolipoprotein occurred with lipid vesicles containing 85% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine and 15% 1,2-dipalmitoyl-sn-glycero-3-phospho-1-glycerol or 1,2-dipalmitoyl-sn-glycero-3-glycerol. Fluorescence measurements on the apolipoprotein indicated that the trypsin-resistant side chains were in a relatively hydrophobic environment, and that the wavelength of maximum fluorescence emission was not changed upon the binding of lipid.

The results suggest that the principal mode of interaction between the apolipoprotein and lipids of surfactant is hydrophobic bonding. The most extensive binding occurs with lamellar lipids in a gel phase, which is expected to have inhomogeneities in packing density due to the presence of acidic phospholipids or other glycerolipids. The role of Ca⁺⁺ in this interaction has not been yet determined. Although it is not needed to effect the binding of the lipids and apolipoprotein, it does influence the physical state of the complex, and possibly the stoichiometry of lipid to protein. Some of the processes mediated by Ca⁺⁺ in this interaction may be analogous to those observed in membrane fusion. Thus, Ca⁺⁺ probably causes a change in the lamellar phospholipids into domains, inducing vesicular disruption and fusion. This lipid aggregates on the hydrophobic sites on the protein, thereby forming high molecular weight reassembly complexes.

The physicochemical processes important in the maintenance of the stoichiometry and structure of pulmonary surfactant are largely unknown. This material contains several phospho and neutral lipids and one or more specific proteins (1), and forms in aqueous solvents a unique lamellar structure ("tubular myelin") (2). Recent results indicate that calcium plays an important part in the formation of this structure (3).

and that the structure is disrupted in solutions with EDTA (4). Certain of the surface properties of natural surfactant are also changed after chelation of calcium, and there are alterations in the distribution of protein to lipid when the material is subjected to high speed centrifugation in density gradients.1 Thus, the structure, physical properties, and function of pulmonary surfactant are intimately related, and appear to be closely dependent upon a unique interaction among the individual components.

In previous publications (5, 6) we examined how certain of the lipids found in relatively high concentrations in surfactant were capable of interacting with the major apolipoprotein of surfactant ("apolipoprotein A") to form lipid-protein complexes. We found that mixtures of lipids containing DPPC readily formed reassembly particles with this protein, but that the physical properties and stoichiometry of the resulting lipoprotein were dependent upon the presence of both calcium and phosphatidylglycerol. Mixtures of DPPC and DPPG, in solutions containing calcium, formed highly aggregated complexes with the apolipoprotein which readily sedimented at relatively low gravitation forces; DPPC without DPPG was capable of interacting with the apolipoprotein but the amount of lipid forming large protein-lipid complexes was much less. We suggested that calcium might be involved in the reassembly complex in three ways. 1) It might induce a change in the structure of the protein; 2) it might serve as an ionic bridge between the phosphatidylglycerol and the protein to facilitate electrostatic binding; or 3) it might induce local stress discontinuities in the planar bilayer of the lipid and thereby affect the lipid-protein interaction by promoting local structure fluctuations (7). The experiments reported in this paper examine these possibilities. The major emphasis is directed to the purported ionic contribution of calcium, and the results of a number of interaction experiments with and without calcium are described. We have also examined the importance of the physical state of the lipid, principally as to whether the lipid needs to be above or below its phase transition to effect reassembly. Finally, in a much more limited manner, we have studied whether certain of the conformational properties of the protein, as revealed by intrinsic fluorescence, are changed either by calcium or after binding to lipid.

MATERIALS AND METHODS

Recombination Studies—Pulmonary surfactant was isolated from canine lavage fluid by isopycnic density gradient centrifugation (8).

1 B. J. Benson, personal communication.

2 The abbreviations used are: DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DODG, 1,2-dioleoyl-sn-glycerol; DOPC, 1,2-dioleyl-sn-glycerol; DPPG, 1,2-dipalmitoyl-sn-glycerol; DSPC, 1,2-distearoyl-sn-glycerol-3-phosphocholine; PMG, 1-palmitoyl-sn-glycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine.
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The protein components were dissociated from the surfactant lipoprotein using lithium diiodosalicylate and solvent partition. Apolipoprotein A was further purified by affinity chromatography using 10-ml columns of Cibacron blue F3GA coupled to agarose gel (Affi-Gel blue, Bio-Rad). The procedure has been described in detail (6). Polyacrylamide gel electrophoresis of the purified preparations was carried out in 1% sodium dodecyl sulfate under reducing conditions (10). The apolipoprotein was labeled with [14C]iodoacetamide using a slight modification of the method of Hunter and Greenwood (9). Lipids were purchased from either Sigma or Serday (London, Ontario) and were of the highest purity available. All were checked for homogeneity by silicic acid thin layer chromatography under conditions capable of detecting about 5% impurity. [3H]palmitic and [14C]cholesterol were obtained from New England Nuclear. Greater than 98% of the radioactivity migrated in TLC with phosphatidylcholine. The lipids used for the interaction studies were dispersed in aqueous buffers by the method of Batzer and Korn (10) and were sonicated briefly. Details of the preparation of these dispersions have been given (6). We used two buffers for most of the experiments: buffer A (0.1 M NaCl, 3 mM CaCl2, 5 mM sodium borate, pH 7.4) and buffer B (0.1 M NaCl, 10 mM EDTA, 5 mM sodium borate, pH 7.4).

The interaction of the apolipoprotein with the liposomes was carried out by mixing 26 µg of apolipoprotein A containing 1 apomolecule to 1300 µg of lipid and mixing the solution in 0.7 ml of either buffer A or buffer B. This range of lipid to protein in prior studies (6) resulted in lipid-protein complexes whose stoichiometries were largely independent of lipid concentration. The [3H]apolipoprotein used in the experiments was greater than 90% precipitated by cold 5% trichloroacetic acid. About 85% of the counts were equilibrated with the same buffer (11). Fluorescence was measured in a Turner model 111 filtered fluorometer using a primary filter with a narrow band pass of 405 nm and a secondary filter passing light of 495 nm. 0.3 ml of the intact vesicles was placed in a fluorescence microcuvette and the fluorescence was recorded for 10 min. A separate aliquot of vesicles was mixed with 0.1 ml of 2% Triton X-100 and the release of the trapped dye was estimated from the increase in fluorescence. The amount of additional amount of Triton X-100 did not increase the fluorescence, indicating complete lysis of the vesicles. Vesicles prepared in buffer B were mixed with either apolipoprotein or with Ca2+, and the fluorescence increment was expressed as the per cent of that obtained with Triton X-100. In most cases there was a considerable fluorescence increase in the control of the materials in the cuvette and had placed it in the fluorometer. This was followed by a slower increase which extended over a 30-min period. We could not, therefore, follow the complete kinetic profile of the reaction. Instead, our data are expressed as the amount of dye released after 30 min, normalized to the amount released by Triton X-100.

Properties

Apolipoprotein A-The preparation of purified apolipoprotein A migrated as one major band and two minor bands, as shown in Fig. 1. The homogeneity of most preparations was estimated by densitometry at about 90%. The protein does not react with antisera directed to dog serum, and has an amino acid composition characterized by relatively large amounts of glycine, hydrophobic amino acids, and acidic groups. Sueishi and Benson (12) have published the amino acid composition of the same protein isolated by different methods; our own results are very similar.

Electrophoresis under reducing conditions indicates that the minimum molecular weight of the protein is about 35,000 to 40,000. Under nonreducing conditions (but in sodium dodecyl sulfate) some of the protein migrates with an apparent molecular weight of 70,000 to 80,000, suggesting the formation of a dimer. Most of the protein, however, is found at the interface between the stacking and separating gel. The protein is eluted in the void volume from a calibrated column of Sephadex G-300 (Pharmacia Fine Chemicals) swollen in 0.01 M sodium borate, pH 7.1, containing 0.1 M sodium chloride. It is likely, therefore, that the apolipoprotein is comprised of eight or more subunits under the conditions used in the reassembly experiments, and its molecular weight is greater than 300,000.
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Recombination Experiments—The separation of bound and free constituents in velocity gradients has been described before (6). The lipoprotein reassembly complex, formed in calcium, is highly aggregated, and is easily sedimented away from the unbound lipid and apolipoprotein, which are recovered at the top of the centrifuge tube. The method is not adequate to distinguish bound and free reactants when the interaction occurs in buffer B (10 mM EDTA) since in this solvent extensive aggregation of the lipid does not occur after binding of the apolipoprotein, and the protein-lipid recombinants are not separated from the unbound constituents. For these experiments, we have used isopycnic density gradient centrifugation, whose effectiveness can be seen in Fig. 2. The results of an interaction experiment between DPPC/DPPG (85:15 weight %) suspended in buffer B and the apolipoprotein are shown (Fig. 2, right). The details of the reaction conditions and the centrifugation are described in the legend to the figure. Unbound protein is sedimented to the bottom of the tube and is easily separated from protein in combination with lipid. Bound and unbound lipid fractions can be discerned, but the reaction stoichiometry of the recombinant particle is difficult to determine with accuracy because of the partial overlap between bound and unbound lipid. We tried a variety of density gradient parameters but were unable to improve upon this separation. However, our principal emphasis in these experiments is on the extent of involvement of the protein, and we feel that this was accomplished accurately. We carried out some recombination experiments in buffer A (3 mM Ca²⁺) and separated the bound and free constituents in isopycnic density gradients. The results are also seen in Fig. 2. The separation of bound and free protein is similar to that in the EDTA experiments, and the resolution of bound and free lipid is improved. A limited number of experiments in buffer A were done using isopycnic centrifugation to separate constituents, and the results were similar to those found with velocity gradients.

We investigated whether the lipid to protein stoichiometry of the recombinant material was relatively constant when the material was recentrifuged under differing conditions. In one experiment, we formed the lipoprotein reassembly particle between apolipoprotein A and DPPC/DPPG, and recovered the lipoprotein from a velocity gradient. This material was then either (a) placed on a velocity gradient with a narrower density range than the first or (b) placed on a sucrose density gradient and centrifuged to isopycnic density. The experimental procedure is described in greater detail in the legend to Fig. 3. The overall lipid to protein ratio of the original recombinant was 355. This material was localized in a relatively narrow band in isopycnic centrifugation, and had a lipid to protein ratio of about 365. With recentrifugation in a second velocity gradient there was a greater spread of the material, but the lipid to protein stoichiometry of the individual fractions was relatively constant with an average of 310. It is likely, therefore, that the distribution of the material in this gradient is reflecting heterogeneity in size rather than that of density or composition.

In a second experiment (not shown) we formed the recombinant between DPPC/DPPG/POPC/cholesterol and the apolipoprotein at 37 °C, and isolated the recombinant from a velocity gradient. This material was diluted with an equal volume of buffer A, incubated for 30 min at 37 °C, and then recentrifuged on a velocity gradient. The lipid to protein

![Fig. 1. Polyacrylamide gel electrophoresis of apolipoprotein A purified from canine surfactant.](http://www.jbc.org/)
stoichiometry of the original material was 149; after recentrifugation it was 151. The only radioactive counts detected in the gradient were found in the position of the recombinant band.

To further assess the possibility that the complexes might be due to a nonspecific entainment of the protein by particulate lipid we carried out a series of experiments measuring the association of bovine plasma albumin (two times crystallized, fatty acid-free) with several mixtures of lipids. The results are shown in Table I. The percentages of albumin isolated as lipid-protein complexes were far less than those obtained with the apolipoprotein (Table II), suggesting that nonspecific entainment might account for less than 15% of the bound apolipoprotein with most of the lipids. Somewhat greater amounts of albumin were bound by DSPC/DPPG than with the other lipids, and the results of the interaction experiments between apolipoprotein A and DSPC/DPPG should be considered in that regard.

The experiments investigating the interaction between apolipoprotein A with lipids were done with 11 mixtures in buffer A. The results are compiled from three to 13 different experiments with each mixture, with the concentration of each lipid varying from about 1 to 2 mM. We found that the amount of bound apolipoprotein, expressed as a percentage of the total recovered, and the amount of bound lipid tended toward a constant value as the concentration of lipid increased. Our results, therefore, are expressed in this manner and are shown in Table II. The lipids used in these experiments are listed in the order of the weight percentage of disaturated lipid in the mixture. We have further grouped the mixtures into those with and without phosphatidylglycerol. We will discuss these groups separately.

All lipid mixtures which contained both DPPG and saturated phosphatidylycholine bound apolipoprotein. The mixture of DSPC/DPPG bound the most protein (but note the effects with albumin, Table I) closely followed by DPPC/DPPG. Both lipid mixtures were comprised of 100% disaturated phospholipid: the distearoyl mixture, however, would be expected to have a phase transition temperature about 19 °C higher than that of the dipalmitoyl (13) unless this relationship was significantly disturbed by the 15 weight % DPPG in both mixtures. The amount of bound apolipoprotein was directly related to the amount of disaturated species in the mixtures which contained DPPG, and even small decreases in disaturated content resulted in lesser amounts of bound protein. Very small amounts of apolipoprotein were bound by vesicles comprised of lipid whose phase transition temperature was well below that of the temperature of the interaction.

Thus only 12% of the apolipoprotein was bound at 37 °C by DOPC/DPPG; adding cholesterol in a molar amount approximately equal to half the phosphatidylycholine resulted in little change. The extent of lipid involvement in the reassembly complexes paralleled that of the apolipoprotein in that the amount of bound lipid decreased with decreasing amounts of disaturated phospholipid. There was, however, a significant difference between DSPC/DPPG and DPPC/DPPG in that the lipid with the higher melting point formed lipoprotein recombincants with greater affinity than the others, even though the total amount of disaturated phospholipid was identical. Cholesterol may also have affected the interaction.

DPPC/DPPG/cholesterol involved more lipid in reassembly than did DPPC/DPPG, as did DOPC/DPPG/cholesterol compared with DOPC/DPPG.

The results obtained with the lipid mixtures not containing DPPG are in the lower part of Table II. The complex formed with DPPC has a relatively low lipid to protein ratio compared with that formed with DPPC/DPPG. Only 6% of the DPPC is bound in the recombincant lipoprotein, an amount comparable to that of the unsaturated phosphatidylycholines. How-

**Table I**

| Lipid                  | Bound protein % of total | Lipid            | Bound protein % of total |
|------------------------|--------------------------|------------------|----------------------------|
| DPPC/DPPG (80/15)*     | 27.2 ±2.0                | DPPC/DPPG (65/15)| 8.2 ±3.4                   |
| DPPC/DPPG/cholesterol (77/13/10) | 11.0                   | DPPC/DPPG/POPC/cholesterol (63/9/19/9) | 5.0 ±0.2                   |
| DPPC/DPPG/POPC/cholesterol (64/27/9) | 0                      |                 |                            |

* Weight per cent.

**Fig. 3.** The stability of the lipid-apolipoprotein recombinant with recentrifugation under differing conditions. 39 μg of apolipoprotein A were mixed for 30 min at 37 °C with 2.8 mg of DPPC/DPPG in 2.4 ml of buffer A. 1.0 ml of the material was transferred to each of two density gradients prepared as continuous sucrose gradients ranging from 4 to 17% sucrose (ρ = 1.016 to 1.064), poured over a 1-ml cushion of 40% sucrose, and centrifuged for 15 min at 15,000 rpm (A). The recombinant lipoprotein was recovered, diluted to 1 ml with buffer A, and placed on either a sucrose gradient of 8 to 17% sucrose (ρ = 1.029 to 1.064) over 1 ml of 40% sucrose or 8 to 24% sucrose (ρ = 1.029 to 1.089). The 8 to 17% gradient was centrifuged at 15,000 rpm for 60 min (B). The 8 to 24% gradient was centrifuged at 25,000 rpm overnight to effect a separation by isopycnic density (C). The lipid to protein stoichiometries of individual points comprising the majority of material in the bands are shown as molar ratios, assuming a molecular weight for the protein of 35,000. ◆ protein; ○, lipid.
ever, DPPG is clearly not required for extensive interaction between all of the lipids and protein. DPPC/DPG bound the largest amount of apolipoprotein of any mixture of lipid, and DPPC/PMG showed an affinity comparable to that of the DPPC/DPPG mixtures and DPPC/DPPG/cholesterol mixtures. DOPC/DODG did not form any detectable recombina
tant, consistent with the behavior of the dioleoylphosphatidylcholine when mixed with phosphatidylglycerol or with phosphatidylglycerol and cholesterol.

The binding of the apolipoprotein by lipids in buffer B is shown in Table III. The amount of lipid involved in the complex is not listed as we feel that the resolution of bound and free lipid in the isopycnic gradients was not adequate for

### Table II

| Lipid                   | Disaturated | Protein (%) | Lipid (%) |
|-------------------------|-------------|-------------|-----------|
| With DPPG               |             |             |           |
| DSPC/DPPG (85/15)*      | 100         | 80 (7.5)    | 71 (11.4) |
| DPPC/DPPG (85/15)       | 100         | 68 (2.6)    | 21 (2.9)* |
| DPPC/DPPG/cholesterol   | 90          | 68 (2.6)    | 58 (16.9) |
| (77/13/10)              |             |             |           |
| DPPC/DPPG/POPC/cholesterol (63/9/19/9) | 72      | 56 (5.6)    | 14 (1.9)  |
| DOPC/DPPG/cholesterol   | 16          | 14 (0.5)    | 7 (1.2)   |
| (67/16/7)               |             |             |           |
| DOPC/DPPG (85/15)       | 15          | 12 (0.8)    | 3 (0.6)   |
| Without DPPG            |             |             |           |
| DPPC (100)              | 100         | 37 (2.1)    | 6 (1.7)   |
| DPPC/DPPG (85/15)       | 100         | 87 (4.5)    | 34 (3.6)  |
| DPPC/PMG (85/15)        | 85          | 65 (5.7)    | 48 (8.4)  |
| DPPC/POPC/cholesterol   | 64          | 41 (3.4)    | 4 (0.3)   |
| (64/27/9)               |             |             |           |
| DOPC/DODG (85/15)       | 0           | 0 (0)       | 0 (0)     |

* Weight per cent.

The recombinant materials formed with DPPC/DPG could be separated by isopycnic gradient centrifugation into two fractions as shown in Fig. 2. The percentages of bound protein and lipid in the fraction with a high protein to lipid ratio are comparable to those in the lipid-protein complex which readily sediments in velocity gradients, and the combined results are shown in this table. The stoichiometry of the fraction with a low protein to lipid ratio could not be determined because of its incomplete separation from the unbound lipid. The additional protein recovered in this fraction, expressed as a percentage of total recovered protein, was 25 (2.0). In one isopycnic experiment using DPPC, we also observed a second fraction of relatively low density which was widely dispersed in the gradient and which appeared to represent recombinant material with heterogeneous protein to lipid stoichiometry. All other lipid-protein recombinants migrated as single fractions.

### Table III

| Lipid                   | Bound protein (mean ± SE) |
|-------------------------|---------------------------|
|                         | 3 mM Ca²⁺ 10 min EDTA    |
|                         | % of total               |
|                         |                           |
| With DPPG               |                           |
| DPPC/DPPG (85/15)       | 66 (2.5)                  | 81 (2.5) |
| DPPC/DPPG/POPC/cholesterol (85/9/19) | 66 (5.6) | 67       |
| DOPC/DPPG (85/15)       | 12 (0.8)                  | 0 (0)    |
| Without DPPG            |                           |
| DPPC (100)              | 37 (2.1)                  | 35 (6.0) |
| DPPC/DPPG (85/15)       | 87 (4.5)                  | 62 (0.7) |
| DOPC/DODG (85/15)       | 0 (0)                     | 0 (0)    |

In contrast, mixing this dispersion
of lipid with the same amount of albumin induced a much more modest aggregation. When vesicles formed in buffer B were mixed with apolipoprotein A, very little change in light scattering was apparent, as also seen in Fig. 4. Adding 14 mM calcium, however, induced a significant change in physical state. Vesicles prepared in buffer B without apolipoprotein became partially aggregated upon addition of 14 mM calcium, but this process could be further supplemented with apolipoprotein.

About 10% of the dye entrapped in vesicles of DPPC/DPPG, formed in buffer B, was released by adding apolipoprotein. In contrast, Ca²⁺, in concentrations of 0.7 and 9 mM, effected the release of over 70% of the trapped dye. The increases in fluorescence in 30 min were comparable when the vesicles were mixed with Ca²⁺, with and without apolipoprotein.

The rates of adsorption of DPPC/DPPG and apolipoprotein, allowed to interact in either buffer A or B for 30 min at 37 °C and then mixed with the respective buffer to provide a subphase concentration of 10 μg/ml total lipid and 0.5 μg/ml of total protein, are shown in Fig. 5. There was no demonstrable change in surface tension for about 30 min of the lipid-apolipoprotein dispersion suspended in buffer B; in contrast, the components in buffer A lowered interfacial tension almost immediately. DPPC/DPPG mixed with apolipoprotein in buffer B adsorbed more rapidly than did the lipid alone; the recombiant in buffer A, however, had a rate of adsorption comparable to that of the lipid in the same buffer. All films lowered surface tension to less than 10 dynes/cm when the surface area was compressed to about 20% of its maximum, indicating that the adsorbed material included lipid.

Electron micrographs of DPPC/DPPG vesicles, processed with negative staining, are shown in Figs. 6 and 7. Vesicles formed in buffer B were spherical and generally intact with diameters varying from 22 to 35 nm. Many of the vesicles appear to have collapsed on themselves, possibly in response to the osmotic stress induced by the evaporation of the fixative solution. The thickness of these units was about 9 nm, suggesting that they were comprised of two closely opposed lipid bilayers. The vesicles were relatively stable, and electron micrographs of this dispersion taken after 30 and 60 min in buffer B at 37 °C were nearly identical with that shown in Fig. 6. The electron micrographs of the dispersion mixed with apolipoprotein showed some aggregation of the vesicles, together with multilayer stacking of the lamellae and the formation of heterogeneous multilayer configurations. The micrograph shown in Fig. 6 was taken of a mixture incubated for 60 min at 37 °C. Micrographs of mixtures incubated for shorter times were similar.

DPPC/DPPG formed in buffer A is shown in Fig. 7. When mixed with stain within 1 min after formation, the lipid appeared as regular segments varying in length from 26 to 44 nm. These segments were about 4 to 5 nm in thickness, about half of that observed for the thickness of the vesicles of the same lipid in buffer B. Within 30 min there was evidence of aggregation, the stacking of the segments into multilayer structures, and the formation of closed vesicular structures. The morphology of the dispersion at 60 min was dominated by spherical multilayer structures ranging in diameter from 17 to 44 nm, although the microscopic fields were more heterogeneous than those observed for the lipid dispersed in buffer B. In contrast, DOPC/DPPG (85:15) formed homogeneous intact vesicles of about 18-nm diameter in both buffers.

DPPC/DPPG mixed with apolipoprotein in buffer A formed highly aggregated multilayer structures within 1 min after formation. The fields were very heterogeneous, but multilayer stacks interdispersed with the 26 to 44 nm segments were predominant. Within 30 min, the material was almost completely aggregated into large structures without regular size or morphology. A few fields indicated that these large units were comprised of heterogeneous multilayer formations.

Fluorescence Experiments on Protein Structure—The apolipoprotein dissolved in buffer B had a maximum emission of fluorescence at 330 nm, indicating a relatively apolar environment around the tryptophen residues (14). Adding 1.5 mM calcium induced a shift in wavelength of about 1 nm, as determined by comparisons of the normalized spectra which crossed with no change in band width, and an increased fluorescence yield of 5%. Increasing calcium to 3 mM did not induce additional changes in the spectrum. When the vesicles of DPPC/DPPG, formed in buffer A, were mixed with the
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**FIG. 7.** Electron microscopy of DPPC/DPPG with and without apolipoprotein A suspended in buffer A at 37 °C. The negative staining was carried out as described in the legend to Fig. 6. A, lipid without protein fixed within 60 s of preparation; B, lipid without protein fixed after 30 min; C, lipid with apolipoprotein A fixed within 60 s after preparation; D, lipid with apolipoprotein fixed after 30 min.

apoipoprotein under conditions known to effect an interaction, there was no further blue shift. Similarly, there was no change in the spectrum when the protein was mixed with lipid vesicles prepared in buffer B.

**DISCUSSION**

The data presented in Table III show that calcium is not required to effect recombination of apolipoprotein A with phospholipids. Six mixtures of lipids were tested for their ability to bind the protein in the absence of Ca²⁺; with all mixtures, comparable amounts of protein were bound irrespective of whether the buffers contained 3 mM Ca²⁺ or 10 mM EDTA. Furthermore, this relationship held irrespective of whether the lipids contained phosphatidylglycerol. Thus, it is unlikely that ionic bridges mediated by calcium play an important part in the interaction between these lipids and apolipoprotein A.

The most significant factor in this recombination relates to the amount of fully saturated phospholipid in the dispersed lipid. In Table II it is seen that the association of protein is related to this variable for all lipid mixtures which contain DPPG. We have attempted to relate these differences in composition to changes in phase transition temperature, as measured by light scattering (15). For all but DPPC and DPPC/DPPG, however, obvious phase transitions could not be detected (data not shown). It is probable that the extent and proportion of gel domains in the lipid bilayer arrays increase as the proportion of fully saturated phospholipid increases. Since binding is enhanced with these lipids it is likely that recombination is primarily driven by hydrophobic interactions between the apolipoprotein and lipid in the gel state. DOPC/DODG, containing no saturated phospholipid, bound no apolipoprotein.

Maximal binding of the apolipoprotein, however, was not obtained using 100% DPPC, but rather with DPPC mixed with 15% DPPG or DPDG. From Table II, it is seen that DPPC bound 37% of the apolipoprotein with 6% lipid in a fraction with a relatively high protein to lipid stoichiometry; DPPC/DPPG bound twice that amount of protein and over four times the amount of lipid. Similar differences can be seen by comparing DPPC/DPPG/POPC/cholesterol with DPPC/POPC/cholesterol; the former mixture of lipids is more effective for the interaction. Furthermore, lipids containing DPDG and PMG formed reassembly lipoproteins with larger proportions of lipid and protein than did those having DPPG. Thus, mixing glycerolipids with dissimilar head groups tended to increase the amount of bound protein, even when the fatty acid compositions of the lipids were identical and ionic interactions were not possible. We think that DPDG, PMG, and DPPG could exert their effects by inducing inhomogeneities in the lipid lamellar structure, or by segregating into domains with adjoining gel-liquid phases. In this way DPPG, DPDG, and PMG may act as perturbants to the planar bilayer gel phospholipid, providing localized regions of instability (7).

Swaney (16) and Massey and colleagues (17) have proposed that the rate of reaction and the stoichiometry of the reaction product, formed by apolipoprotein A-II from high density plasma lipoproteins and dimyristoylphosphatidylcholine, are dependent upon structural defects induced by adjoining regions of gel and liquid crystalline lipid. Considerable indirect evidence supports the concept of phase separations in planar bilayers (see, for instance, Ref. 18). DPDG and PMG have markedly different physical properties than DPPC (19, 20), and it seems likely that they would induce discontinuities in the lamellar packing of the gel, even though no one, to our knowledge, has directly demonstrated this. It is also clear that
mixtures of acidic phospholipids and phosphatidylcholines segregate into domains when dispersed in buffers containing calcium (21), or form nonlamellar phases (22). There is less information on the physical state of such mixtures in EDTA. Aggregation, flocculation, or fusion does not occur in the times covered by these studies (Ref. 23 and our own observations), and the temperatures and enthalpies of the phase transitions for 1,2-diacyl-sn-3-phosphoglycerols are very similar to those of the analogous phosphocholines as much as 20 °C below their major thermal transitions (7). It seems possible, therefore, that such "defects" would exist in lamellar arrays comprised of a mixture of phosphatidylglycerol and phosphatidylcholine at a temperature only 4 °C lower than the phase transition temperature, and that their importance in the interaction between lipids and proteins needs to be considered.

From these observations we postulate the following sequence of events in the binding of apolipoprotein A to phospholipids dispersed in aqueous solutions. Apolipoprotein A interacts with lamellar arrays of phospholipids by hydrophobic bonding, with particular preference for ge-state phosphocholines which may have defects in their packing arrangement. When calcium is not present, this binding is not associated with a significant lysis of single bilayer vesicles. However, in the presence of calcium, vesicular instability induced by a separation of lamellar phases (21) or the formation of a nonlamellar phase (22) results in the disruption and fusion of the lipid vesicles, either around the hydrophobic apolipoprotein or with itself, to form lipid-protein and fused multilayered lipid structures. The extent to which lipoprotein recombinants are produced instead of aggregated lipid structures will depend upon the relative free energy of protein-lipid hydrophobic bonding as compared with the energetics of the self-association of the lipid (28). With apolipoprotein A and saturated phosphatidylcholines (mixed with phosphatidylglycerol), the formation of lipoproteins is favored; with albumin and the same lipids, there is little protein-lipid association.

In three experiments we attempted to duplicate this hypothetical sequence. We mixed apolipoprotein with DPPC/DPPG in buffer B at 37 °C for 30 min. We then added sufficient CaCl₂ to bring the calcium concentration to 28 mM, and incubated the suspension an additional 60 min. Bound and free constituents were separated on isopycnic density gradients. The results of one experiment are shown in Fig. 8. The calcium significantly altered the distribution of bound and free lipid and the stoichiometry of the lipoprotein recombinant. In the three experiments, the amount of associated lipid increased in the presence of calcium by an average of 34%. This represents a conservative figure since the resolution of bound and free lipid in buffer B gradients was not optimal and we are probably overestimating the amount of lipid bound to protein in this buffer compared with that in buffer A. Thus, we conclude that calcium promotes the formation of recombinant particles with relatively high lipid to protein stoichiometry, consistent with our observations in previous experiments (6).

Our information on the structure of the protein is limited, but it is all consistent with the hydrophobic interaction of an apolar surface of the apolipoprotein with perturbed regions of the bilayer. Thus, the minimum molecular weight of the protein is about 40,000 in sodium dodecyl sulfate-mercaptoethanol electrophoresis, but is greater than 300,000 in solution. The tryptophan residues are in a highly apolar environment in the free protein, suggesting an internalized hydrophobic region. Furthermore, there is a small blue shift of the fluorescence spectrum upon binding Ca²⁺, suggesting that the tryptophans are shifted to an even more apolar region. The role played by calcium in its interaction with the apolipoprotein is difficult to assess. Calcium is not required for the protein to bind the lipid, although it may affect the stoichiometry of the recombinant complex (6). We have presented arguments suggesting that calcium induces important changes...
in the structure of the lipid; the effects of any conformational changes that it may induce in the protein are less evident.

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J. Biol. Chem. 1983, 258:10672-10680.

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