Interaction between Hepatic Microsomal Membrane Lipids and Apolipoprotein A-I*

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Incubation of apoprotein A-I (apo-A-I), the major protein component of human high density lipoprotein, with rat liver microsomal membranes under conditions of elevated pH and ionic strength leads to the production of a soluble protein:lipid complex (A-I/MM complex). The A-I/MM complex, as purified by density gradient centrifugation and agarose column chromatography, possesses a lipid composition similar to that of plasma high density lipoproteins, but markedly different from that of recombinant particles prepared with synthetic lipids. The A-I/MM complex constitutes a more physiological recombimant particle than can be formed using synthetic lipids and may be a suitable model for the newly assembled intracellular high density lipoproteins. Incubation of the erythrocyte plasma membranes with apo-A-I under the same conditions as used with microsomal membranes fails to generate any lipid:apolipoprotein complexes. This membrane specificity for forming soluble lipoprotein complexes suggests that the microsomal membranes possess a unique feature, possibly their lipid composition, which render them particularly suitable to serve as lipid donors to the apoproteins which are undergoing assembly within the endoplasmic reticulum/Golgi organelles.

Recent studies have shown that the major protein of high density lipoprotein, apo-A-I,1 is synthesized in the liver and intestine (1-4). It remains obscure, however, how the HDL reaches its fully assembled state, especially given the heterogeneity of this lipoprotein species in plasma. In the rat intestine, the A-I appears to be secreted into lymph in three or more forms—one as a surface component of the chylomicron particle, a second as a discoidal product containing A-I and E, with a density characteristic of the HDL, and the third a spherical particle with an apoprotein composition similar to rat plasma HDL, containing A-I, E, and A-IV (5, 6). Studies utilizing perfused rat livers suggest that the liver also secretes a discoidal product in the HDL density range (3, 7); the major protein component of this particle is the E apoprotein, although the A-I and A-IV proteins are also present. This composition is quite different from that of plasma HDL and suggests that there may be substantial redistribution of the apoprotein moieties of these particles following their secretion by the liver.

Studies of HDL biosynthesis in rat and chick liver (8-10) have shown that protein:lipid complexes are formed by the time that the newly synthesized apolipoproteins have reached the Golgi apparatus. The source of the lipids incorporated into the nascent apoproteins has not been defined. Lipoprotein lipid could be derived from lipids coordinately synthesized with the apolipoproteins or from lipids pre-existing in the membranes of the endoplasmic reticulum/Golgi organelles. If the latter situation is, in fact, the case, then it would remain to be determined whether or not lipid uptake requires the mediation of a lipid transfer factor similar to those factors in the plasma or cytosol which have been previously described (11, 12).

Investigations of the association of lipid with the apoproteins of human HDL (13-15) have established that this may occur readily with some phospholipids; however, many phospholipids, including those which figure prominently in the composition of plasma lipoproteins and cell membranes, combine with apoproteins poorly or not at all in the absence of detergents or high power sonication. Furthermore, the presence of elevated amounts of cholesterol in synthetic phospholipid bilayers has been shown to repress recombination between lipids and apoproteins, suggesting that cholesterol composition of natural membranes might also influence their ability to donate lipids for the formation of protein:lipid complexes.

The present studies were undertaken to determine whether in vitro incubation of the A-I apolipoprotein with cellular membranes, in the absence of lipid synthesis, can result in the acquisition of membrane lipids to form soluble lipid:apolipoprotein complexes.

MATERIALS AND METHODS

Preparation of Labeled Apoprotein—HDL was isolated from the d 1.063-1.21 g/ml fraction of human plasma by preparative ultracentrifugation (16). Lipoproteins were lyophilized and delipidated with ethanol/ether to obtain apo-HDL (17). The apo-A-I was purified from apo-HDL by gel chromatography using a Sephadex G-150 column (2.5 x 190 cm) eluted with 0.1 M Tris-HCl, pH 8.2, containing 3 M guanidine hydrochloride (18). The apo-A-I fraction so isolated was labeled with [3H]acetic anhydride (19) by multiple additions of the [3H]acetic anhydride dissolved in methyl-Cellulose. The radiolabeled protein, denatured in 6 M guanidine hydrochloride, was dialyzed to remove excess label and to renature the apoprotein. As calculated from the specific activity, less than 1 mol of acetyl group was introduced per mol of apoprotein. The electrophoretic patterns on SDS gels of the radiolabeled protein, both with and without cross-linking,
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appeared identical with those of the unmodified protein, suggesting that the hydrodynamic and self-associative properties were unchanged by this minimal degree of modification.

**Rat Liver Subcellular Membrane Isolation and Characterization**—Livers obtained from Sprague-Dawley strain male rats (150–200 g) were homogenized in 3 volumes of 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0, per g of liver. To this mixture was added to tissue which was minced on an ice-cold glass plate and the suspension was homogenized in a glass homogenizer. The pellet (nucleation fraction) separated by centrifugation at 10,000 rpm for 90 min. All centrifugation was performed at 4 °C. The supernatant was used for the isolation of the membrane fraction, as described by Chowdry and Arias (20). Plasma membrane was isolated from the nucleation fraction pellet according to the method of Touster et al. (21). Specific activities of 5’ nucleotidase and alkaline phosphodiesterase I were used as markers to estimate the yields of plasma membrane fractions. Glucose-6-phosphatase and nucleoside diphosphatase activities served as markers for microsomal membrane and cytochrome oxidase was a marker for mitochondria. The assay used for 5’ nucleotidase was that of Arkesteijn (22); the assay for glucose-6-phosphatase was that of the De Duve et al. (23); nucleoside diphosphatase and alkaline phosphodiesterase activities were determined by the method of Beaufay et al. (24). The enzyme assays were performed on freshly isolated subcellular membrane fractions.

Bovine and human red cell ghosts were prepared by the method of Kant and Steck (25).

**Incubation of Native Membranes with Apo-A-I**—For reconstitution studies using intact native membranes, a known amount of labeled apo-A-I was added to either the microsomal or plasma membrane fractions to achieve a phospholipid/apoprotein ratio of 100:1 (w/w). To this solution was added an equal volume of 3 mM triethanolamine-HCl, pH 9.7, which contained 0.185 M KBr and 2 mM phenylmethylsulfonyl fluoride (Buffer A) with a resultant pH of 9.3; in some cases, 0.01% butylated hydroxytoluene was added as a lipid antioxidant. The incubation was performed at 37 °C for 24 h in the shaking water bath. After incubation, the samples were centrifuged for 30 min in a Model 235A Fisher microcentrifuge. The pellet and the supernatant were recovered and the radioactivity in each was determined by liquid scintillation spectrometry. In some cases, an aliquot of [3H]cholesterol was introduced by drying a chloroform solution of [3H]Apo-A-I with microsomal membranes at neutral pH.

**Isolation of A-I/Microsomal Membrane Complexes**—To isolate the recombinant complex from incubation of A-I with microsomal membranes, an aliquot of the nucleation fraction was determined by the centrifugation in a microcentrifuge to pellet the membranes. The supernatant was layered on top of a KBr density gradient. The gradient was prepared in SW41 centrifuge tubes by successively layering 3.0 ml of d 1.21 KBr, 4.0 ml of d 1.125 KBr, and 4.3 ml of d 1.063 KBr (26). The tubes were centrifuged at 15 °C for 90 h at 40,000 rpm in a Model L5-50 ultracentrifuge. The pellet and the supernatant were recovered and the radioactivity in each was determined by liquid scintillation spectrometry. In some cases, a tracer label of [3H]cholesterol was added by drying a chloroform solution of [3H]cholesterol on the walls of a test tube and incubating the microsomal membranes overnight at 4 °C in the tube.

**Interaction of A-I/Membrane Preparations**—To form protein:lipid complexes by incubating apo-A-I with microsomal membranes at neutral pH produced inconsistent results with generally poor yields of complexes. The possibility was considered that the failure to readily form complexes might be due to the closed, cytosolic-side-out nature of the microsomal vesicles, with the resultant lack of access to the cisternal side of these membranes (34). A recent report by Higgins and Pigott (34) has given evidence for differences in lipid composition between the cisternal and cytosolic sides of these membranes. Based on their results, we altered our protocol by adding Buffer A at a 1:1 proportion to the membrane (final concentrations, 0.5 M triethanolamine-HCl, pH 9.3, 0.08 M KBr, 1 mM phenylmethylsulfonyl fluoride) to disrupt the microsomal vesicles. In our initial experiments, a tracer for membrane lipids was incorporated into the membranes by incubating them with radioactive cholesterol to allow us to evaluate lipid incorporation by the A-I protein.

In one such experiment, the labeled microsomal membrane...
fraction was disrupted with the high pH buffer and incubated with either labeled apo-A-I, unlabeled apo-A-I, or buffer at 37 °C for 5 h. This incubation mixture was then centrifuged to pellet the membrane components and the supernatant was applied to an agarose A-5m column (Fig. 1). For the first of these samples, A shows that the majority of the [3H]apo-A-I label elutes at a volume of 120 ml; this fraction contains the A-I/" complex. This elution volume is identical with that for synthetic complexes of A-I and dimyristoyl phosphatidylcholine at low lipid/protein ratios (26) and greater than the elution volume observed for plasma HDL. The cholesterol label is seen to elute in the region of the A-I/MM complex as well as in a later peak where salts are found to elute. It should also be noted that in these experiments the protein radioactivity in the supernatant and membrane pellet was measured following incubation. It was found that 99% of the protein was located in the supernatant fraction and applied to the column, indicating that A-I does not become incorporated into the membrane matrix, but remains in the supernatant either in lipided or unlipided forms.

As a control for the possible effects of covalent modification of the protein, unlabeled protein was used; the elution profile in Fig. 1B shows an identical distribution of labeled cholesterol, suggesting that protein modification did not influence the experimental outcome. When the labeled membrane fraction was incubated with buffer alone (C), the only fraction containing the cholesterol label was the salt volume peak, indicating that this peak contained cholesterol which desorbed from the membrane even in the absence of added protein.

Fractions from the elution profile in Fig. 1B were pooled in 30-ml portions and phospholipid phosphorus and Lowry protein were determined. The phospholipid was found mainly in the two peaks containing tritiated cholesterol, with virtually no phospholipid or protein in any fractions preceding the region where the A-I/MM complex elutes. These data reinforce our belief that these incubations yield essentially only a single protein/lipid complex.

In some experiments, the incubation buffer was changed to 0.1 M Na2CO3, pH 11.3, since Howell and Palade found this to be an effective method for emptying the contents of rat liver Golgi vesicles (9). This method was at least as effective as inducing lipid:protein complex formation with the microsomal membrane fraction as Buffer A (data not shown), but was so disruptive of the membrane structures that separation of A-I/MM complexes from residual membrane fragments was more difficult.

Specificity of the Membrane:apo-A-I Interaction.—Since the apolipoproteins are amphipathic molecules which are thought to have detergent-like properties, the possibility was considered that apo-A-I could nonspecifically remove lipids from any membranes with which it might come in contact. For comparison with the microsomal membranes, we isolated the plasma membrane from liver cells for study of interactions with apo-A-I. This membrane was not nearly as effective at donating lipids to apo-A-I as the microsomal membranes, although such studies were not definitive because of the presence in all preparations of some cross-contamination by intracellular membranes which could account for the small amount of complex formation which was observed.

As a source of a more homogeneous plasma membrane fraction, erythrocyte ghosts were prepared and used for incubations with apo-A-I. In one such study, microsomal vesicles or erythrocyte ghosts were incubated with [14C]apo-A-I at a 10:1 ratio of membrane phospholipid to protein (w/w) in Buffer A for 20 h, the membranes were pelleted by centrifugation, and the supernatant was layered on a discontinuous KBr gradient. The distribution of A-I radioactivity in collected fractions is shown in Fig. 2. These results demonstrate that all of the A-I is bound to microsomal lipids in a complex with a density of 1.14. By contrast, [14C]A-I incubated with erythrocyte ghosts shows no tendency to complex with lipid since protein radioactivity is found to elute in the region where apo-A-I is observed to elute under these conditions.

Since similar results were obtained when these incubations were performed with 0.1 M sodium carbonate, pH 11.3, this suggests that reactivity of the microsomal membranes is due to the disruptive effect of elevated pHi or ionic strength, rather than to the chemical nature of the buffer per se and that the nonreactivity of erythrocyte plasma membrane under equivalent conditions may be due to its particular composition or structure.

Characterization of the A-I/MM Complex.—Since disruption of the microsomal vesicles could result in the release of membrane proteins with associated lipid, a major concern was for the purity of the A-I/MM particles formed during the incubation. To this end, SDS-gel electrophoresis was per-
formed on samples of the incubation mixture (Fig. 3, lanes 5 and 6) and on the lipoprotein fraction isolated by density gradient centrifugation (Fig. 3, lanes 3 and 4). The isolated fraction can be seen to contain almost exclusively the A-I protein; i.e. there is little evidence of the major membrane proteins seen in the incubation sample, with the possible exception of a low molecular weight band seen at the bottom of the gel. In order to ascertain whether the band seen in the upper region of lanes 3 and 4 might be albumin, rat serum albumin was run in lanes 1 and 2. Samples applied to lanes 1, 3, and 5 were reduced with mercaptoethanol to demonstrate that albumin changes mobility upon reduction whereas the upper band in lane 4 does not. We believe that this band is a covalent dimer of the A-I protein, possibly resulting from cross-linking due to lipid oxidation. If, however, we assume that this band is a contaminant from the membrane, a maximum estimate of mass, based on relative staining, would be 15%. Because of molecular weight differences, this would correspond to an 8% contamination on a molar basis; considering the stoichiometry of the particles, we would estimate that a maximum of one particle in seven would contain such a contaminating protein. For this reason, we feel that protein components from the membranes themselves are not obligatory for the formation of the A-I/MM complex.

The stoichiometry of the protein:lipid complex was studied at the same time by the technique of chemical cross-linking with dimethyl suberimidate; this approach has proven very helpful in establishing the quaternary structure of other protein:lipid recombinant particles (26). Lane 8 of Fig. 3 shows such a cross-linked sample and lane 7 shows the cross-linked self-associated forms of this protein, ranging from monomers to pentamers. The A-I/MM complex appears quite homogeneous in its quaternary structure and it appears that each particle contains two A-I chains.

The isolated A-I/MM complex was also analyzed chemically to define its composition (Table I). The percentage of protein was reproducibly found to be about 50%, which is similar to that reported for serum HDL and to that reported for nascent HDL isolated from perfused rat livers. The protein content is, however, considerably higher than the value of 29% or less reported for complexes of A-I and dimeristoyl phosphatidylcholine by numerous investigators (e.g. Refs. 26, 36, and 37).

The distribution of lipids in the A-I/MM complex appears to closely resemble that of the parent membrane fraction (Table II). Considering the distribution of phospholipid species, there does not seem to be any evidence for selective interaction with specific lipid domains in the membrane possessing a unique lipid composition. The phospholipid distribution is similar to that of plasma HDL, except that plasma HDL has more phosphatidylcholine and less phosphatidylethanolamine than the A-I/MM complex.

**TABLE I**

| Protein          | Cholesterol | Phospholipid | Triacylglycerol |
|------------------|-------------|--------------|-----------------|
|                  | Free        | Ester        |                 |
| **A-I/MM**       | 51.2 ± 1.6  | 41.1 ± 1.4   | 44.5 ± 3.1      | ND              |
| Human HDL<sup>6</sup> | 55         | 12           | 23              | 12              |
| Human HDL<sup>4</sup> | 41         | 16           | 30              | 5               |
| Rat nascent HDL<sup>2</sup> | 39         | 4            | 40              | 6               |
| DMPC:A-I<sup>4</sup> | 29         | 71           |                 |                 |
| Dimer complex    | 29         | 71           |                 |                 |
| Trimer complex   | 17         | 83           |                 |                 |

<sup>a</sup> Average of three determinations, each of which was performed in duplicate.

<sup>b</sup> Cholesterol ester was not detectable in the A-I/MM complex.

<sup>c</sup> Triacylglycerol was determined only once and found to be very low (<0.5% of total composition).

<sup>d</sup> From Ref. 49.

<sup>e</sup> From Ref. 7; data correspond to nascent HDL isolated from rat liver perfusates for which buffer containing 5,5'-dithiobis(2-nitrobenzoic acid) was used.

<sup>f</sup> Assuming a lipid:protein ratio of 100:1 (mol/mol) for the dimer complex and 200:1 (mol/mol) for the trimer complex (Refs. 26, 35, 36). DMPC, dimyristoyl phosphatidylcholine.
Electron microscopy was also performed on the protein:lipid complex to study its morphology and to ascertain whether this product possessed the discoidal structure ascribed to "nascent HDL" obtained from perfused rat livers (3, 7). Samples of the A-I/MM complex isolated by a combination of density gradient centrifugation and agarose gel filtration revealed spherical particles (Fig. 4). The distribution of particle sizes obtained from these studies is given in Fig. 5; a maximum in the distribution is seen at 9–11 nm with a fairly normal distribution about the mean. When an identical incubation of apo-A-I and microsomal membranes were performed in the presence of 9 mM 5,5'-dithiobis-(2-nitrobenzoic acid) to inhibit lecithin:cholesterol acyltransferase, the appearance of the particles in the electron micrographs was similar to those of samples from which this reagent was excluded; specifically, no rouleaux structures such as those seen with recombinants formed from apo-A-I and synthetic lipids were observed.

Study of the Time Course of Protein:Lipid Complex Formation—Studies of lipoprotein synthesis by the liver have suggested that these particles may be assembled within a period of 30 min. We were therefore interested in determining the time course for the protein-lipid association process in our system. Previous assays of protein-lipid recombination have relied on the clearing of liposome turbidity (13, 26); since no clearing of the membrane suspensions was observed (or expected) under our conditions of incubation, an alternative approach was devised (Fig. 6). Incubations of the microsomal membrane fraction with [14C]apo-A-I were prepared, aliquots were removed at appropriate time points, the membranes were removed by centrifugation, and the supernatant fractions were cross-linked. In this way, the extent of lipid-protein association, as judged by conversion to the dimer structure characteristic of the A-I/MM complex, was determined as a function of the time that the protein was left in contact with the membrane. An autoradiogram of the SDS gel electrophoretic pattern shows the progressive shift in cross-linking pattern between that of the apoprotein alone and the dimer pattern (Fig. 6, lane 7). As judged by the reduction in intensity of the monomer band, and increase in dimer band, formation of a protein-lipid complex appears to approach completion in less than 1 h. When this approach was employed to study the reaction with erythrocyte ghost membranes, it appeared that there was little or no alteration in the initial apoprotein cross-linking pattern even after 24 h (Fig. 6, lanes A–G).

**Table II**

Lipid composition of hepatic microsomal membrane, A-I/microsomal membrane recombinant, and human plasma HDL

| Lipid species          | Microsomal membrane | A-I/MM complex | Human HDL* |
|------------------------|---------------------|----------------|------------|
| Total cholesterolb     | 5.0 ± 1.0           | 8.4 ± 2.9      | 37         |
| Phospholipidf          | 95.0 ± 1.0          | 91.6 ± 6.4     | 55         |
| Phosphatidylcholinec   | 58.3 ± 2.9          | 58.5 ± 2.3     | 74         |
| Phosphatidylethanolaminec | 21.2 ± 3.5       | 14.1 ± 3.5     | 3.3        |
| Sphingomyelinc         | 14.8 ± 0.8          | 12.4 ± 1.2     | 15         |
| Phosphatidylinositolc  | 2.7 ± 0.6           | 4.0 ± 0.8      | 2.4        |
| Phosphatidylserinec    | 2.3 ± 0.7           | 5.5 ± 1.9      | 1.9        |
| Lysophosphatidylcholinc | 1.8 ± 0.4           | 5.5 ± 0.9      | 2.0        |

* From Ref. 50.

† Average of three determinations ± S.E.

‡ Average of six determinations ± S.E.

§ Comparison of MM with A-1/MM; n.s. = not significant.

Fig. 4. Electron microscopy of the isolated A-I/MM complex. An incubation mixture containing [14C]apo-A-I and microsomal membranes (1 mg of protein to 10 mg of membrane phospholipid) was incubated at 37 °C for 20 h and applied to a KBr density gradient. Following centrifugation for 96 h, the gradient was fractionated and the tubes containing the protein radioactivity were applied to a column (2.6 × 90 cm) of agarose A-5m. The fraction containing the A-I protein was collected for electron microscopy. Enlargement of the photograph is 150,000 diameters.

Fig. 5. A-I/MM complex particle size distribution. The electron micrographs obtained of the isolated A-I/MM complexes (e.g. Fig. 4) were analyzed by measuring the apparent diameters visualized by negative staining. A total of 550 particle diameters were measured.

Evaluation of Possible Mechanisms for Protein:Lipid Complex Formation—The effect of a high pH environment on Golgi vesicles has been demonstrated to be one of membrane disruption, with partial liberation of vesicle contents, exposure of the inner side of the membranes, and exposure of "free ends" of the membranes (8). In order to evaluate whether the vesicle contents of our microsomal fraction might include a
factor, analogous to the cytosolic phospholipid transfer protein described by Wirtz and Zilversmit (37), we measured the effect of adding back an aliquot of the first extraction of microsomal membrane vesicle contents to an incubation of apo-A-I and erythrocyte ghosts. When the time course was followed and compared to an apo-A-I/erythrocyte ghost incubation to which Buffer A alone was added, the results with both Buffer A alone and with microsomal contents extracted with Buffer A were identical with those presented in lanes A-G of Fig. 6. We conclude from this that the contents of microsomal membrane vesicles do not contain a factor which will promote lipid uptake from the erythrocyte plasma membrane by apo-A-I.

Recombination of Apo-A-I with Extracted Membrane Lipid Liposomes—When membrane lipids, extracted from the microsomal fraction by the Bligh and Dyer procedure (27), were formed into multilamellar vesicles (26) and allowed to incubate with apo-A-I overnight at 37 °C in 10 mM Tris-HCl, pH 7.5, it was found that a complex was readily formed which possessed similar properties to the complex obtained with intact microsomal membranes. These contained 47% protein, 50% phospholipid, and 3% cholesterol. The density of the recombinants was 1.14 g/ml, as judged by separation on salt density gradients by ultracentrifugation. Chemical cross-linking showed that the protein:lipid complexes contained 2 molecules of A-I per particle.

**DISCUSSION**

A primary focus of investigations from this laboratory has been to try to elucidate how lipoproteins might be assembled within the hepatic or intestinal cell. Our previous studies of the formation of soluble lipid:apoprotein complexes using multilamellar phospholipid bilayers constructed from single phosphatidylcholines or binary mixtures of these have indicated that recombination is kinetically limited by the availability of suitable defects in the bilayer and that the optimal conditions for achieving these defects, and hence efficient recombination, occur at the onset of acyl chain melting. It was also found that elevated cooperativity of melting during the phase transition enhances the reactivity toward recombination (14, 15). Lecithins with long acyl chains or with unsaturated bonds react poorly or not at all with apo-A-I or apo-A-II (14, 15). These data suggest that natural membranes, which contain these types of phospholipids, might also be unlikely to donate lipids to apoproteins.

On the other hand, when phospholipid bilayers are constructed with a physiological phospholipid, bovine brain sphingomyelin, reconstitution is readily obtained and is not acutely dependent upon reaction at an identifiable phase transition temperature (38), perhaps due to the heterogeneity of the acyl chain groups. This suggests that compositional differences could play a profound role in determining the reactivity of the bilayer toward abstraction of its lipids by apoproteins.

Studies of interaction between apoproteins and natural membranes have been few in number; it was found by Stein et al. (39) that human apo-A-I was not effective at removing cholesterol from the plasma membrane of ascites cells. On the other hand, it has been known for some time that lipoproteins, including VLDL and HDL, can be found in the Golgi apparatus of the hepatic cell (8, 10, 40). To our knowledge there have not been any studies to determine whether this assembly of proteins and lipids occurs as a result of the ability of the protein moiety to remove lipids from the internal membranes which they encounter post synthetically or whether a more complex mechanism is involved.

The data presented here have demonstrated that a soluble, protein:lipid complex can be generated by incubating apo-A-I with a hepatic microsomal membrane fraction, but only under conditions where the closed, cytosolic-side-out vesicles are disrupted by high pH. The fact that a protein:lipid complex can be formed by incubation with isolated membranes implies that coordinated synthesis of lipids and proteins is not required for lipoprotein assembly. This supports a similar conclusion by Kook and Rubinstein (41) which was based upon differential secretion of labeled lipids and proteins from rat liver slices (41).

It is interesting to note that protein:lipid complex formation could be readily achieved with microsomal membranes but not with plasma membranes from erythrocyte ghosts, even under identical conditions. We have considered the possibility that disruption of the microsomal vesicles releases a factor which promotes lipid transfer to the growing lipoprotein particle. However, addition of supernatant extracts from aliquots of microsomal membrane incubated under our high pH conditions to mixtures of plasma membrane and apo-A-I did not appear to promote lipoprotein formation. We conclude that compositional or structural differences between these membrane types accounts for their difference in reactivity toward apo-A-I. We note the 6–7-fold higher proportion of cholesterol to phospholipid in the plasma membrane compared to microsomal membranes; because we and others have previously shown that elevated levels of cholesterol in synthetic phospholipid bilayers inhibit recombination (38, 42), it is tempting to speculate that the cholesterol content of membranes may play a significant role in mediating their reactivity in this regard. It has been recently shown that sterol depletion of mouse fibroblast cells accelerates the hydrolysis of phosphatidycholines in the membrane by phospholipase A2 (40). Phospholipase-mediated hydrolysis may require a similar insertion into the bilayers as is required for the formation of protein:lipid complexes (15) and thus the cholesterol content of the membrane may play a consistent role in modulating.

**FIG. 6.** Time course of A-I dimerization during incubation with microsomal membranes (lanes 1–7) or with erythrocyte ghosts (A–G). For the first set of samples, 2.1 mg of microsomal membrane (2.1 mg of phospholipid) was mixed at a 1:1 ratio with Buffer A (v/v) and then incubated at 37 °C with 200 μg of [14C]A-I during which timed aliquots were removed. For the second set of samples, 68 μg of [14C]A-I was incubated with 700 μg of erythrocyte ghost membranes under the same conditions. Aliquots were removed at 5 min (lanes 2 and B), 15 min (lanes 3 and C), 45 min (lanes 4 and D), 2.5 h (lanes 5 and E), 7.5 h (lanes 6 and F), and 24 h (lanes 7 and G); cross-linked apo-A-I is shown in lanes 1 and A. Samples were centrifuged to pellet the membranes, cross-linked with dimethyl suberimidate (26), and electrophoresed on a 3–27% polyacrylamide gradient SDS gel.
access to the acyl chain portion of the bilayer.

It should also be noted that recombinants similar to the A-I/MM complex can be generated by incubation of apo-A-I with multilamellar vesicles prepared from the lipids extracted from the microsomal fraction; this suggests that the protein components of the microsomal fraction are probably not involved in mediating the formation of the resultant lipoprotein complex.

Since it has been shown that the intracellular transit time for HDL or VLDL in hepatic cells is on the order of 30 min (8, 10), we attempted to ascertain whether the rate of lipoprotein assembly under our conditions might be compatible with this time frame. Although we did not have a means of determining the time dependence for the appearance of the lipoprotein complex directly, we did employ chemical cross-linking to allow us to measure the change in protein quaternary structure with time (Fig. 6). We found that, by this criterion, the rate of recombination was appreciable and appeared to be substantially completed within an hour. Since we were constrained by practical considerations to using a relatively low ratio of membrane to apo-A-I, this approach could well underestimate the rate of lipoprotein assembly which can be achieved. Thus, it would appear that reconstitution in an in vitro experiment can occur at a rate compatible with the rate of HDL biosynthesis in vivo.

The ability to produce a protein/lipid complex with a density of HDL by incubation with a natural membrane led us to compare its properties not only with "physiological" lipoproteins, such as the nascent HDL, but also with the "nonphysiological" synthetic recombinants which have been prepared from individual lipids. The chemical composition of the A-I/MM complex (Tables I and II) indicates a similarity to plasma HDL in its protein/lipid ratio but a much higher ratio than has been observed in recombinant lipoproteins prepared from synthetic lipids. The high percentage of protein (50%) is consistent with the density of 1.14 g/ml estimated from its flotation behavior in salt gradients (Fig. 2). The size of the particles is also similar to that of plasma HDL as judged by gel filtration elution characteristics and by electron microscopy.

The phospholipid distribution of the A-I/MM complex is essentially identical with the microsomal membrane which served as the lipid donor. The phospholipid species of the A-I/MM complex are reminiscent of the distribution found in either rat or human plasma HDL except that the A-I/MM complex contains significantly more phosphatidylethanolamine (Table II). Howell and Palade (8) have reported that VLDL isolated from rat Golgi contains over twice as much phosphatidylethanolamine as plasma VLDL, but that otherwise the composition of Golgi and plasma VLDL are very similar. We might speculate that during or after transit through the plasma membrane, conversion of phosphatidylethanolamine to phosphatidylcholine is brought about by the action of methyltransferase activity, since this would bring the phospholipid composition of both the Golgi VLDL and our A-I/MM complex close to that of plasma VLDL and HDL, respectively. It should be noted that this phospholipid distribution is much different from plasma membranes, which have a significantly lower proportion of phosphatidylcholine.

By electron microscopy (Fig. 4), the A-I/MM complex as isolated by agarose column chromatography did not give rise to the rouleaux structures which are characteristic of phospholipid:apolipoprotein recombinants, even when the incubations were performed in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid), an inhibitor of the lecithin:cholesterol acyltransferase enzyme. We were surprised by this result since "nascent" HDL particles isolated from perfused rat livers in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid) do show rouleaux. On the other hand, HDL isolated from Golgi contents by Howell and Palade also fail to show rouleaux (8). It is possible that the A-I/MM complex is discoidal but fails to stack, for reasons which are unknown; we often observe variable amounts of stacking in preparations of dimyristoyl phosphatidylcholine-A-I recombinants (38, 44). We did not find measurable amounts of cholesterol or triglyceride in the isolated complex, which would indicate that there could not be a significant amount of core lipids.

It has been shown that the A-I protein is synthesized with an 18-residue signal sequence which is cleaved upon translocation across the microsomal membrane (45). It has been further shown that the A-I protein also contains at its NH₂ terminus a 6-residue "pro-segment" of unknown function which is cleaved following secretion into the plasma (46, 47). Although our studies have been performed with apo-A-I, rather than the proapo-A-I form, we expect that the results obtained would be similar, inasmuch as both forms apparently bind to lipids and, furthermore, a 6-residue segment would be unlikely to have much effect on lipid binding by this protein since proteolytic fragments of A-I also bind lipids (48).

In summary, we have defined conditions under which apo-A-I can combine with the lipids of microsomal membranes to form a lipoprotein particle with many of the properties which would be expected of a nascent HDL. The specificity of the complex formation suggests that the cisternal face of the microsomal membranes, as opposed to the cytosolic side of this membrane or either side of the plasma membrane, is particularly designed to facilitate the assembly of lipoproteins by serving as a lipid donor. The availability of a recombinant containing a physiological distribution of lipids should aid in studies of the biogenesis and metabolism of plasma HDL.

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Note Added in Proof—Recently, a characterization of the small, spherical HDL particles which are observed in patients with lecithin:cholesterol acyltransferase deficiency was published (Chen, C., Applepoge, K., King, W., Glomset, J., Norum, K., and Gjone, E. (1984) J. Lipid Res. 25, 269-282). These particles, which are believed to be of intestinal origin, were found to have the following composition: 59% protein, 35% phospholipid, 2.8% free cholesterol, 1.0% cholesteryl ester, and 2.1% triglyceride. Analysis showed 1.8 A-I molecules per particle and a particle weight of 85,000. In these respects, the small, spherical HDL bear substantial similarity to the A-I/MM particles we obtain in vitro (Table I). The A-I/MM particles differ from the small, spherical HDL in having a somewhat lower density (1.14 g/ml versus 1.23 g/ml) and larger apparent average diameter (100 A versus 60-62 Å). Chen et al. propose that the small, spherical HDL may be "nascent" HDL and may become converted to HDL-sized particles by the action of phosphatidylcholine:lecithin:cholesterol acyltransferase. It appears that our A-I/MM complexes may provide a reasonable in vitro model for the small, spherical HDL and that microsomal membranes may be the source of lipids found in the "nascent" HDL particles.

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