Interaction between Unilamellar Egg Yolk Lecithin Vesicles and Human High Density Lipoprotein†

John V. Chobanian, Alan R. Tall, and Peter I. Brecher*

ABSTRACT: The interaction between unilamellar egg yolk lecithin vesicles and human plasma lipoproteins was studied in vitro as a model system for examining the mechanisms by which vesicles are modified in plasma. Vesicles were prepared by sonication and contained radiolabeled lecithin and small amounts of labeled cholesteryl oleate. Following incubation with plasma at 37 °C the vesicle-associated lipids eluted from Sepharose 4B columns as a smaller particle, similar in size to high density lipoprotein (HDL), and were isolated in the same density range as HDL by ultracentrifugation. A similar transformation occurred following incubation with HDL or apoHDL, but not when vesicles were incubated with purified very low or low density lipoprotein. Detailed studies of the interaction between HDL and vesicles indicated the rate of transformation to be temperature dependent and influenced by the relative concentration of reactants. Cholesteryl oleate was transferred from vesicles to the smaller particle more rapidly than lecithin. Chemical analysis of phospholipid in column eluates indicated complete transformation under optimized conditions, thereby excluding an exchange phenomenon. Protein was found to associate with vesicles during the transformation process. The transfer of lipids from unilamellar vesicles to an HDL-like particle suggests that analogous mechanisms for the mobilization of lipoprotein or membrane-associated lipids may occur in biological systems.

Phospholipid vesicles have been widely used in biological studies as model membranes (Tyrell et al., 1976; Gregoriadis, 1976). The in vivo fate of multilamellar liposomes and unilamellar vesicles has been investigated (Gregoriadis et al., 1974; McDougall et al., 1974; Juliano & Stamp, 1975), but the interaction of such preparations with plasma components is incompletely understood. Our laboratory reported that in vivo administration of unilamellar egg yolk lecithin (EYL)[1] vesicles into rats led to transformation of the vesicles to a different particle with a size and density resembling HDL (Krupp et al., 1976). This in vivo transformation may be mediated by apoproteins since in vitro studies have shown that isolated apoproteins complex with phospholipid dispersions to form discoidal particles (Forte et al., 1971; Atkinson et al., 1976) with a density and size similar to HDL (Scanu & Wisdom, 1972; Assman & Brewer, 1974).

In order to characterize the interaction between unilamellar phospholipid vesicles and plasma lipoproteins, in vitro studies were performed using egg yolk lecithin (EYL) vesicles and human plasma, isolated lipoproteins or apoproteins. The interaction between EYL vesicles and isolated HDL was studied in detail as a function of incubation time, temperature, and concentration.

Materials and Methods

Materials. α-L-Dipalmitoyl-2-palmitoyl[9,10-^{3}H]phosphatidylcholine (specific activity 13 Ci/mmol) was purchased from Applied Science Laboratories, and cholesteryl [1-^{14}C]oleate (specific activity 21.5 mCi/mmol) was obtained from Amersham/Searle Corp. Egg yolk lecithin was purchased from Lipid Products (Surrey, England), and all other lipids were from Applied Science Laboratories, Inc.

Isolation of Lipoproteins. Freshly obtained human plasma from fasted adults was collected in 0.1% EDTA and fractionated by sequential preparative ultracentrifugation at 13 °C in a Beckman L2-65B ultracentrifuge using a 60 Ti rotor. VLDL (d = 1.006 g/mL), LDL (d = 1.006–1.063 g/mL), and HDL (d = 1.063–1.21 g/mL) were isolated sequentially by centrifugation at 114000g for 16, 24, and 48 h, respectively. Solutions were adjusted to the appropriate densities with a 7.57 M NaBr, 0.195 M NaCl, 1 mM EDTA solution (pH 8.5). All lipoproteins were washed once at the highest density and then dialyzed for 48 h against several changes of 0.195 M NaCl, 1 mM EDTA, pH 7.5.

Delipidation of Plasma. Freshly obtained plasma was adjusted to a density of 1.25 g/mL and centrifuged at 114000g for 48 h. The resulting supernatant (d < 1.25 g/mL) and infranatant (d > 1.25 g/mL) were separated and dialyzed against 0.195 M NaCl, 1.0 mM EDTA (pH 7.5) for 48 h. Alternatively, plasma was delipidated by solvent extraction using diisopropyl ether and butanol as described by Cham & Knowles (1976).

Preparation of ApoHDL. HDL was delipidated with chloroform:methanol (2:1 v/v) and washed 3× with anhydrous diethyl ether at 15 °C, according to the procedure of Scanu et al. (1969).

Preparation of EYL Vesicles. Cholesteryl oleate containing vesicles were prepared according to a modification of our previously described procedure (Brecher et al., 1977). A mixture of egg yolk lecithin (60 mg), cholesteryl oleate (600 μg), [^{3}H]dipalmitoyllecithin (12 μCi), and cholesteryl [1-^{14}C]oleate (4.0 μCi) was dissolved in chloroform:methanol (2:1, v/v). The solvent was evaporated under a nitrogen stream, lyophilized for 60 min, and resuspended by vigorous agitation in a 6.0-mL solution containing 0.10 M NaCl, 0.01 M Tris, and 0.02% sodium azide (pH 7.4). The resulting suspension was sonicated for 10 min in a nitrogen atmosphere at 25–35 °C in a water-jacketed sonication cell using a Branson W350 sonifier equipped with a standard 0.5-in. horn at an output setting of 6. The preparation was centrifuged at 130000g for 30 min and the supernatant, hereafter referred to as the standard EYL preparation, was stored at 2 °C and used within 1 week of preparation. The molar ratio of EYL to cholesteryl oleate in the vesicle preparations was about 70:1.

† From the Department of Medicine and Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118. *Received March 3, 1977.* This work was supported by U.S. Public Health Service Grant HL 12869. Peter Brecher is recipient of a Research Career Development Award.

[1] Abbreviations used: EYL, egg yolk lecithin; HDL, high density lipoprotein; apoHDL, the total apoproteins of HDL; VLDL, very low density lipoprotein; LDL, low density lipoprotein; SE, standard error.

0006-2960/79/0418-0180$01.00/0

Incubation Conditions. Aliquots of plasma or lipoprotein fractions were incubated with the designated amounts of EYL vesicle preparations in stoppered 12 × 85 mm test tubes under a nitrogen atmosphere. Total incubation volume was adjusted to 0.5 mL with 0.1 M NaCl, 0.01 M Tris (pH 7.4). Following incubation at the designated time and temperature, aliquots were chilled and applied immediately to Sepharose 4B columns.

Sepharose Gel Filtration. Sepharose 4B columns (0.9 × 60 cm) preequilibrated with 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 0.02% sodium azide were used for most of these studies described. Routinely, a series of four columns was packed simultaneously, used for four to six analyses, and then discarded. Samples (0.3–0.5 mL) were applied to the columns and eluted with the preequilibration buffer at flow rates of 7–9 mL/h. In several experiments, 0.5 mL of an EYL vesicle preparation was initially applied to each column to minimize the subsequent adsorption of phospholipid (Huang, 1969). However, it was found that essentially complete recovery of phospholipid was obtained even on the initial samples under our experimental conditions. All chromatographic procedures were conducted in a cold room at 2–4 °C. The fractions collected from the columns were analyzed directly for radioactivity using a toluene–Triton X-100 scintillation cocktail (Brecher et al., 1976). Double isotope counting conditions were established so that ^{14}C was counted at 45% efficiency with no crossover of ^{3}H into the ^{14}C channel. ^{3}H was counted at 15% efficiency and there was a 14% crossover of ^{14}C into the ^{3}H channel. Data were expressed as cpm after correcting for crossover.

Equilibrium Density Gradient Ultracentrifugation. Linear NaBr gradients were generated with a peristaltic pump and centrifuged at 40 000*g* for 24 h in a SW 56 swinging bucket rotor. The density of individual fractions was determined by weighing 250-μL aliquots. Phosphorus was determined on each fraction by the method of Bartlett (1959) and cholesterol by the method of Rudel & Morris (1973). Protein was determined by the method of Lowry et al. (1951) following precipitation by 10% trichloroacetic acid.

Chemical Analysis. Protein was determined by the method of Lowry et al. (1951) and phospholipid by the procedure of Bartlett (1959). Lipid extracts were obtained from aqueous samples according to the procedure of Folch et al. (1957). Thin-layer chromatography was performed on silica gel plates; individual phospholipids were separated using chloroform:methanol:water (65:25:4, v/v) as developing solvent (system 1), and the major lipid classes were resolved using hexane:diethyl ether:acetic acid (70:30:1, v/v) as developing solvents (system 2).

Electron Microscopy. Fractions were negatively stained with 2% sodium phosphotungstate, pH 7.4, on Formvar-coated Cu grids and sized as described previously (Tall et al., 1977).

Results

Characterization of Lipid Vesicles. The elution profile of the standard vesicle preparation, made by sonicating a mixture of egg yolk lecithin, [^{3}H]dipalmitoyllecithin, and cholesteryl [1-^{14}C]oleate, is shown in Figure 1A. The data are expressed as cpm (corrected for crossover) to simplify graphical representation. Most of the radioactivity applied to the column was included within the Sepharose 4B and eluted as a symmetrical peak. The ratio of labeled phospholipid to cholesteryl oleate remained constant both in the ascending and descending portions of the major peak, consistent with single population of unilamellar vesicles containing both lecithin and cholesteryl oleate. The position of the major peak corresponded exactly

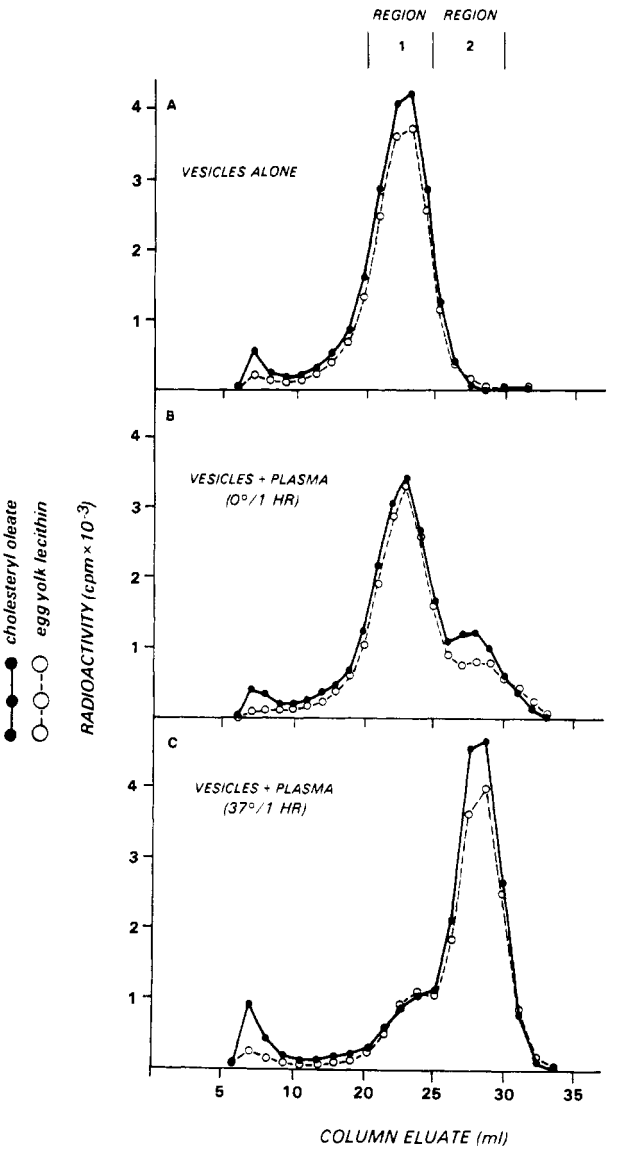

FIGURE 1: Sepharose 4B gel filtration of standard EYL vesicles incubated with human plasma. (A) Radioactive elution profile of the standard vesicle preparation (0.28 mg of phospholipid in 30 μL) containing [^{3}H]lecithin and cholesteryl [1-^{14}C]oleate. Elution profiles of EYL vesicles (0.28 mg of phospholipid with 0.5 mL of plasma) incubated at 0 °C (B) or 37 °C (C).

to that obtained when a preparation of vesicles containing phospholipid but no cholesteryl ester was applied. The vesicle peak was routinely observed to appear between 20 and 25 mL of column eluate, and this region was designated as region 1.

The smaller peak, containing a higher ratio of cholesteryl ester to phospholipid than the main peak, eluting near the void volume, appeared consistently in all preparations tested, and may represent incompletely sonicated multilamellar liposomes. Thin-layer chromatography (system 1) of the vesicle preparation and of eluted fractions in the ascending, peak, and descending portions of the vesicle region indicated lecithin as the only phospholipid present. Thus, lysolecithin was not formed during the sonication procedure.

Interaction of EYL Vesicles with Intact Human Plasma. Figures 1B and 1C show the elution profile of the labeled lipids following incubation of 30 μL of vesicles (0.28 mg of lecithin) with fresh human plasma (0.5 mL) for 60 min at 0 °C (Figure 1B) and 37 °C (Figure 1C). Following incubation at 37 °C, the elution pattern of the labeled lecithin and cholesteryl oleate was altered significantly. A new peak, appearing in a region

eluting closer to the total column volume, was observed, and only a small percentage of the total radioactivity was seen in the region corresponding to the original vesicle preparation. The new peak eluted between 25 and 30 mL, and this region was designated as region 2. Following incubation with plasma at 0 °C, most of the radioactivity remained associated with a particle eluting at the position of unilamellar vesicles (region 1); however, a small but significant amount of both cholesteryl oleate and lecithin did elute at a position corresponding to a smaller particle size (region 2).

In separate experiments it was determined that no hydrolysis of the labeled lecithin or cholesteryl oleate occurred during the incubation procedures. Thin-layer chromatography was performed on lipid extracts from the reaction mixture using solvent systems 1 and 2 (see Materials and Methods). The region corresponding to individual phospholipids, free fatty acids, and cholesteryl esters was scraped into counting vials for scintillation counting. All the radioactivity was associated with either lecithin or cholesteryl ester. The absence of labeled free fatty acid also was confirmed by solvent partition of the original incubation mixtures using benzene:chloroform: methanol and NaOH according to a previously described procedure (Pittman et al., 1975) which effectively separates free fatty acids from other major lipid classes. Incubation of the vesicle preparation alone at 37 °C for 60 min resulted in a Sepharose elution pattern identical with that shown in Figure 1A. Thus, the data suggested that a plasma component mediated a temperature-dependent transfer of lecithin and cholesteryl oleate from vesicles to a different particle of smaller size.

Interaction of EYL Vesicles with Delipidated Human Plasma and Isolated Lipoproteins. Experiments were performed to determine whether plasma lipoproteins were involved in the temperature-dependent effect described above. Initially, plasma was delipidated by ultracentrifugation at a density of 1.25 g/mL, and the supernatant and infranatant were collected separately. The fraction of $d > 1.25$ g/mL contained only trace amounts of cholesterol and phospholipid. When aliquots of both fractions were incubated with EYL vesicles containing labeled cholesteryl oleate at 37 °C for 60 min and applied to Sepharose 4B columns only the supernatant ($d < 1.25$ g/mL), which contained plasma lipoproteins, caused the radioactivity to elute in region 2.

To determine which of the major lipoprotein classes were involved in the transfer process, vesicles were incubated with individual lipoprotein fractions isolated by sequential ultracentrifugation. The amounts of lipoprotein added to each incubation mixture were adjusted to approximate the amount present in 0.5 mL of normal plasma. Following incubation at 37 °C for 60 min, the samples were analyzed by Sepharose 4B gel filtration (Figure 2). No transfer of radioactivity into region 2 occurred in 1- (Figures 2A and 2B) or 16-h incubations with either VLDL or LDL. In the presence of intact HDL, almost all the cholesteryl oleate eluted in the region corresponding to peak 2. However, labeled lecithin was only partially transferred and was found both in region 1 and region 2 (Figure 2C).

The products of lipoprotein–EYL vesicle interaction were further characterized by ultracentrifugation techniques. Following incubation of LDL, VLDL, and HDL with EYL vesicles, mixtures were subjected to preparative ultracentrifugation (Table I). Control vesicles were found primarily between densities 1.006 and 1.063 g/mL. When HDL was present, 82% of the vesicle radioactivity was transferred into the HDL density range in contrast to the almost complete

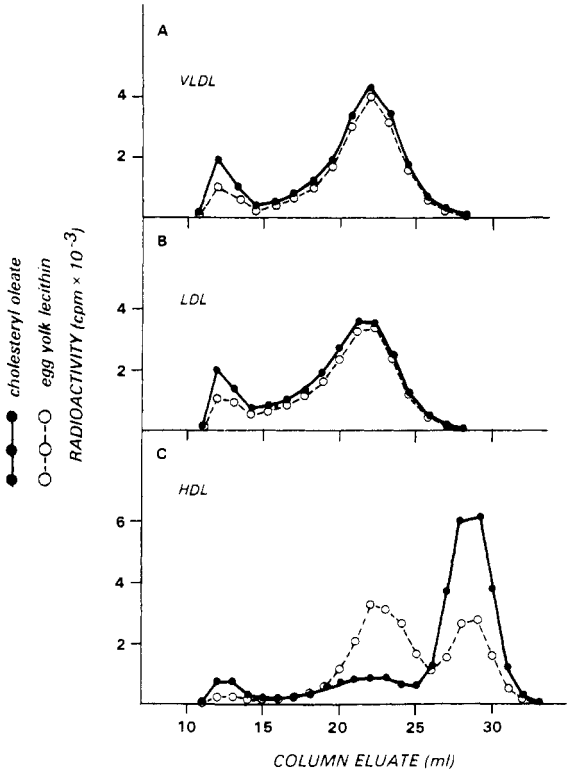

FIGURE 2: Sepharose gel filtration of standard EYL vesicles incubated with isolated lipoprotein fractions. The standard EYL vesicles (0.28 mg of phospholipid) were incubated at 37 °C for 1 h with the individual lipoproteins. (A) VLDL (0.05 mg of protein), (B) LDL (0.4 mg of protein and (C) HDL (0.7 mg of protein). Gel filtration of isolated lipoproteins showed that VLDL eluted predominantly in the void volume while LDL eluted in the same region as the standard vesicles (not shown).

Table I: Percent Distribution of [^{3}H] Lecithin and [^{14}C] Cholesteryl Oleate Following Density Ultracentrifugation of Labeled Vesicles Incubated with Isolated Lipoproteins[a]

	ultracentrifuge fraction					
	$d < 1.006$		$d = 1.006$–1.063		$d = 1.063$–1.21	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
vesicles alone	6	6	93	93	1	1
vesicles + VLDL	26	15	70	83	4	2
vesicles + LDL	9	9	91	91	0	0
vesicles + HDL	0	0	18	20	82	80

[a] Standard EYL vesicles (0.28 mg of phospholipid) were incubated at 37 °C for 17 h with the designated lipoprotein (VLDL, 0.08 mg of protein; LDL, 0.32 mg of protein; HDL, 1.1 mg of protein) in a total volume of 0.5 mL. Aliquots of the incubation mixtures were centrifuged in a 40.3 rotor at densities of 1.006, 1.063, and 1.21 for 24 h at 102 000*g*. Radioactivity was determined in the upper, middle, and lower portions of the ultracentrifuge tube.

absence of radioactivity in this density range following incubation with VLDL or LDL. After incubation with VLDL, radioactivity did appear in the $d < 1.006$ g/mL fraction, suggesting an interaction between VLDL and the vesicles. This interaction, which could represent exchange of lipid between VLDL and the vesicles, was not examined further in these studies.

To characterize further the HDL-like product, incubated mixtures of EYL vesicles and HDL_3 were subjected to equilibrium density gradient ultracentrifugation within the HDL density range (Figure 3). Control vesicles floated at the top of the gradients while control HDL_3 formed a peak

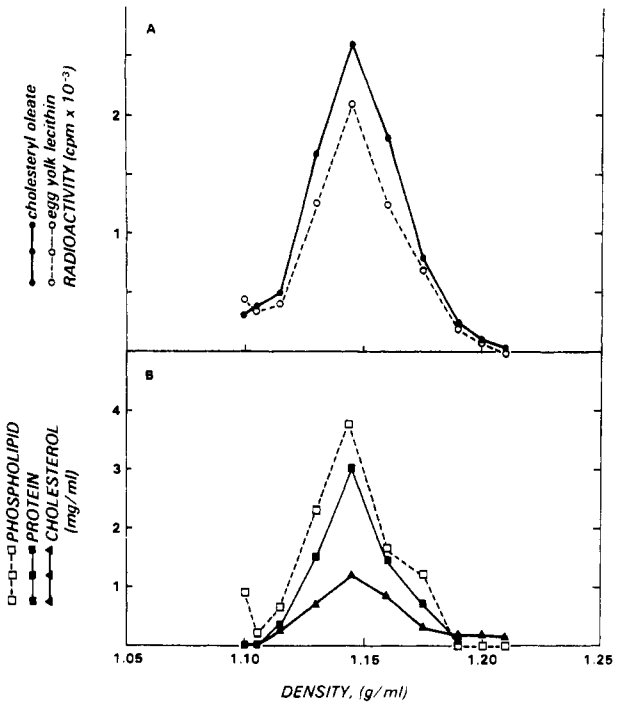

FIGURE 3: Equilibrium density gradient ultracentrifugation of EYL vesicles and HDL_3 incubated at 37 °C for 60 min. The reaction mixture contained HDL_3 (2.6 mg of protein) and standard EYL vesicles (1.75 mg) and was uniformly distributed throughout the gradient tube prior to centrifugation as described in Materials and Methods. Fractions were analyzed for radioactivity (A) and chemically (B).

at d = 1.15 g/mL (data not shown). These experiments showed transformation of vesicle radioactivity into particles of similar density to HDL_3. Phospholipid, cholesterol, and protein banded in a single peak of density = 1.14 g/mL similar in position to control HDL_3. The transfer of phospholipid radioactivity to an HDL-like particle could not be accounted for by exchange processes since there were no appreciable amounts of phospholipid on the tops of the gradients.

Interaction of EYL Vesicles with HDL. The nature of the HDL–vesicle interaction was studied in greater detail by systematically varying the ratios of the reactants and determining the characteristics of the transformation at different incubation times and temperatures. In these experiments, the column eluates were analyzed chemically both for phospholipid and protein in addition to determining the distribution of radioactive lecithin and cholesteryl oleate.

Figure 4 shows the isotopic and chemical analysis of column eluates following incubation of the vesicle preparation (0.94 mg of lecithin) with a relative excess of HDL (2.8 mg of protein, 1.5 mg of phospholipid). Figure 4A illustrates the isotopic data after incubation at 0 °C for 60 min and indicated the presence of intact unilamellar vesicles eluting in the region of peak 1. Following incubation at 37 °C for 60 min (Figure 4B), most of the labeled cholesteryl oleate but only about 50% of the labeled phospholipid was found in region 2. The pattern was similar to that shown previously in Figure 2C and again illustrated the selective transfer of the cholesteryl ester. However, when incubation was prolonged to 16 h (Figure 4C), both labeled lipids were localized predominantly in region 2. Incubation of EYL vesicles alone at 37 °C for 16 h produced a radioactive elution profile identical with that shown in Figure 1A.

Additional insight into the reaction mechanism was obtained by chemical analysis of the eluates. Figure 4D shows that, following the 0 °C incubation, most of the HDL protein was found in region 2. A small but significant amount of protein was found in region 1. Chromatography of HDL alone showed no protein in region 1 (data not shown). Phospholipid eluted as two distinct peaks and correlated quantitatively to a summation of phospholipid analysis performed on column eluates of either vesicles or HDL applied in separate experiments. Following incubation at 37 °C, little change in the protein distribution was observed, although significantly less phospholipid was present in region 1 after 60-min incubation (Figure 4E). Almost all the phospholipid was present in region

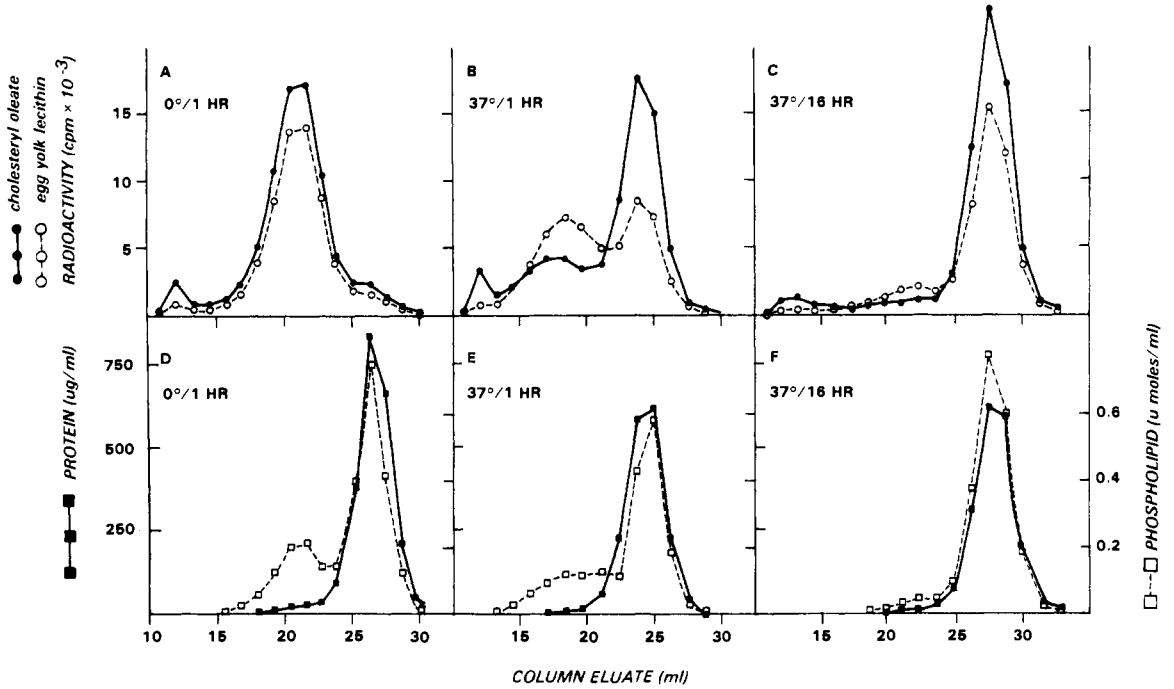

FIGURE 4: Sepharose gel filtration of standard EYL vesicles incubated with intact HDL. All incubation mixtures contained EYL vesicles (0.94 mg of phospholipid) and HDL (2.8 mg of protein). Incubations were performed at 0 or 37 °C for the times indicated, and the column eluates were analyzed for radioactivity (A–C) and chemically (D–F).

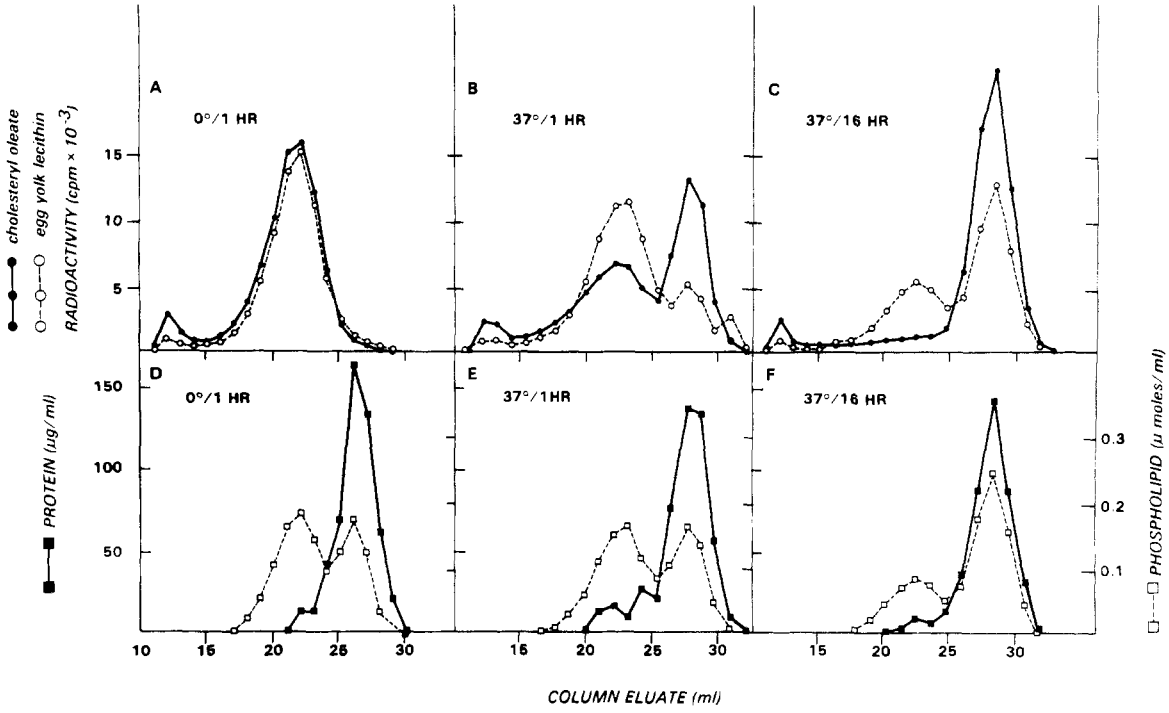

FIGURE 5: Sepharose gel filtration of standard EYL vesicles incubated with intact HDL. All incubation mixtures contained EYL vesicles (0.94 mg of phospholipid) and HDL (0.7 mg of protein). Incubations were performed at 0 and 37 °C for the times indicated, and the column eluates were analyzed isotopically (A–C) and chemically (D–F).

2 following the 16-h incubation period (Figure 4F). The data clearly show a net transfer of vesicle-associated lecithin to a smaller particle similar in size to HDL. The specific activity of the phospholipid in the peak 1 region did not change appreciably during the experiments, indicating that exchange was not a major factor in the interaction. The almost complete disappearance of phospholipid from region 1 after prolonged incubation indicated disruption of the original vesicles occurred.

A second series of incubations was performed in which the amount of HDL was decreased by a factor of 4 relative to the vesicles, so that each incubation tube contained EYL vesicles (0.94 mg of lecithin) and 0.7 mg of HDL-associated protein. Figure 5 (A through F) shows the data from column eluates according to the protocol followed in Figure 4. The isotopic data for the incubations (Figures 5A–C) indicate the presence of intact vesicles at 0 °C (Figure 5A), transfer of about 60% of the cholesteryl oleate and 15% of the labeled lecithin by 1 h at 37 °C (Figure 5B), and complete transfer of the labeled cholesteryl ester following incubation at 37 °C for 16 h, in spite of the presence of significant amounts of labeled lecithin still present in region 1 (Figure 5C). This incomplete transfer of vesicle-associated lecithin even after 16-h incubation contrasted with the complete transfer observed at this incubation time when the relative amount of HDL was fourfold greater (Figure 4C). Chemical analysis of the phospholipid in the corresponding fractions (Figures 5D–F) indicated that the labeled lecithin was a reliable indicator of vesicle-associated egg yolk lecithin since the decreases in total phospholipid in the vesicle region corresponded quantitatively to the loss of radioactivity from that region. Most of the protein remained in the HDL region (region 2), but significant amounts were present in region 1 in all the experiments shown, particularly after incubation at 37 °C for 60 min (Figure 5E). Aliquots of the fractions from regions 1 and 2 were analyzed by electron microscopy following negative staining with 2% phosphotungstate. A uniform population of circular particles with a mean diameter of 217 + 4 Å (mean SE) were routinely observed, whereas, in region 2, circular particles with sizes ranging between 70 and 80 Å were present.

A third series of experiments was performed following a similar protocol to that described above but employing incubation conditions where the vesicle concentration was increased relative to HDL, so that the final incubation mixtures contained 3.6 mg of vesicle-associated EYL and 0.7 mg of protein present in HDL. Figure 6 (A through F) shows the data from these experiments. Analysis of radioactivity in the column eluates indicated no transfer of radioactive lecithin or cholesterol oleate during 60-min incubation at 0 or 37 °C (Figures 6A and 6B). However, after 16-h incubation at 37 °C, most of the cholesteryl oleate and about 35% of the phospholipid was found in region 2 (Figure 6C). Chemical analysis of phospholipid was again consistent with the radioactive data, indicating net transfer of lecithin from vesicles to the HDL region after 16-h incubation (Figure 6F), but no appreciable transfer at the other incubation conditions (Figures 6D and 6E). Protein determinations revealed a significant amount of protein in the vesicle region in all cases.

Since a relatively high concentration of protein was observed in region 1 in the experiments shown in Figure 6, it was of interest to determine whether lipid components derived from HDL also were present in this region. Lipid extracts were obtained from fractions in region 1 and aliquots applied to silica gel thin-layer plates for development in solvent systems 1 and 2. There were only trace amounts of sphingomyelin, cholesterol, and cholesteryl ester compared with control experiments analyzing equivalent amounts of intact HDL. Furthermore, the specific activity of phospholipid in region 1 remained unchanged during the experiments, suggesting that the protein present in region 1 was predominantly apoHDL, unassociated with HDL-derived lipid.

Interaction of Apoproteins with Vesicles. The role of apoproteins from HDL in mediating the observed transformation of vesicles was examined. Plasma was delipidated by solvent extraction according to the procedure of Cham &

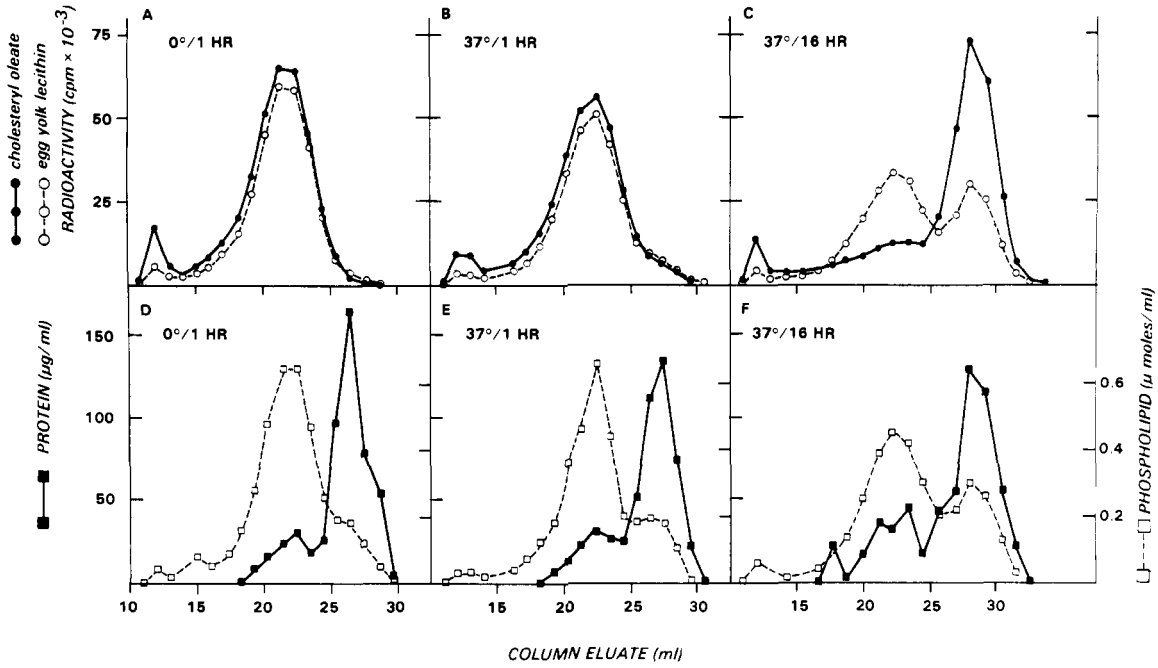

FIGURE 6: Sepharose gel filtration of standard EYL vesicles incubated with intact HDL. All incubation mixtures contained EYL vesicles (3.6 mg of phospholipid) and HDL (0.7 mg of protein). Incubations were performed at 0 and 37 °C for the times indicated, and the column eluates were analyzed isotopically (A–C) and chemically (D–F).

Knowles (1976), resulting in a lipid-free preparation containing apoproteins from all lipoprotein classes. Incubation of this preparation with EYL vesicles resulted in formation of a radioactive peak in region 2 containing labeled lecithin and cholesteryl oleate (Figure 7A). The presence of a poorly defined peak in region 1 and at the void volume also was apparent, possibly caused by traces of organic solvent which were not removed during the delipidation procedure. Figure 7B shows the elution pattern of vesicles incubated in the presence of purified apoHDL. An amount of apoHDL equivalent to the apoprotein present in the incubations summarized by Figure 4 was added to the incubation mixture. All the radioactivity was found in region 2 associated with the apoprotein, clearly demonstrating that apoHDL can effect the transformation under study.

Discussion

The interaction of EYL vesicles with intact human HDL was shown to result in the transformation of the vesicles to smaller particles similar in size to HDL. In a previous study, we had shown that an analogous transformation occurred under in vivo conditions when vesicles were injected into rats, or when incubated with rat plasma in vitro (Krupp et al., 1976). In this study we have demonstrated this transformation occurred when EYL vesicles were incubated with human plasma; we have indicated that HDL is the active plasma component mediating the transformation and, by using the purified HDL preparations, we have shown the dependency of the reaction on incubation time, temperature, and the relative ratio of the reactants.

Isotopic analysis of the distribution of labeled lecithin and cholesteryl oleate from column eluates showed that a similar process occurred when EYL vesicles were incubated with whole plasma, isolated HDL, or purified apoHDL, indicating an important role for the apoHDL in the transformation. Isolated apoproteins from HDL do interact with phospholipids under different experimental conditions. Incubating EYL vesicles with HDL apoproteins, Assman & Brewer (1974) observed the formation of lipid protein complexes having a size and

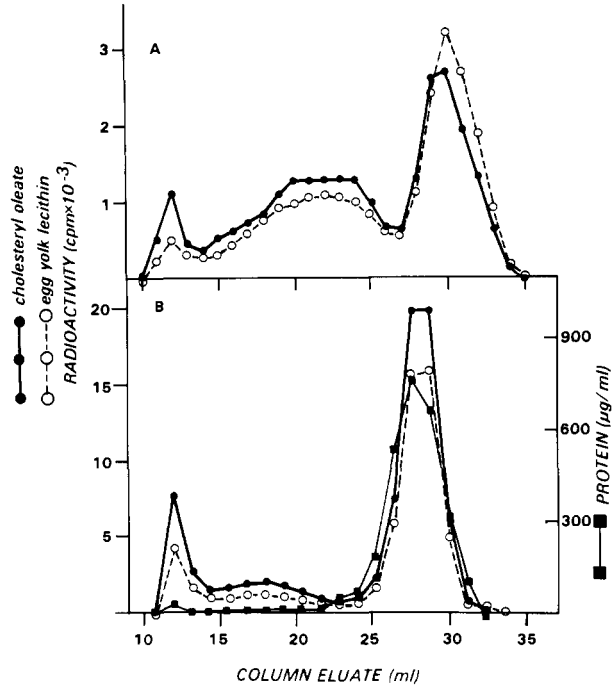

FIGURE 7: Sepharose gel filtration of standard EYL vesicles incubated with apoproteins from plasma or HDL. (A) EYL vesicles (0.38 mg of phospholipid) were incubated at 37 °C for 1 h with plasma delipidated by extraction with organic solvents (35 mg of protein); (B) EYL vesicles (0.94 mg of phospholipid) were incubated at 37 °C for 16 h with purified apoHDL (2.5 mg of protein).

density similar to HDL. Discoidal particles with a density similar to HDL were formed when apoproteins from HDL were sonicated with EYL (Forte et al., 1971). The formation of a complex between EYL vesicles and apoCIII, predominantly a VLDL-associated apoprotein, was demonstrated following brief incubation of the purified apoprotein with a relative excess of EYL vesicles (Morrisett et al., 1974) and in a related study apoCIII was shown to disrupt dimyristyllecithin vesicles, resulting in the formation of apopro-

tein–lecithin complexes with a micellar rather than a vesicular structure (Novosad et al., 1976). It is possible that an analogous process, involving the disruption of EYL vesicle particles in the presence of intact HDL or whole plasma, occurred in the present study.

Binding of apoproteins to lecithin stabilized triglyceride emulsions were reported following in vitro incubation of the emulsion particles with intact or delipidated plasma (Havel et al., 1973; Sata et al., 1976). Tall & Small (1977) have shown that intact HDL interacted with dimyristoyllecithin liposomes to form discoidal apoprotein–phospholipid complexes similar to those reported using purified apoproteins. Our data indicate that the transformation of EYL vesicles to particles of smaller size by intact HDL was an apoprotein-mediated process and was dependent on the ratio of vesicles to available apoHDL.

A possible mechanism for the transformation described in this study is that apoprotein, loosely associated with HDL, attached to EYL vesicles subsequent to collision between HDL and a vesicle particle, and the resulting apoprotein–vesicle complex underwent breakdown to smaller particles when a sufficient ratio of apoprotein to phospholipid was attained. Collision between particles is a diffusion regulated process, dependent upon temperature and the concentration of reactants. The existence of an apoprotein–vesicle complex was indicated by chemical analysis of column eluates, showing significant amounts of protein associated with the vesicle region following incubation at 0 and 37 °C particularly when intact HDL was incubated with a relative excess of EYL vesicles (Figure 6).

The stability of the apoprotein–vesicle complex and its subsequent breakdown to smaller particles may depend on temperature and the apoprotein:phospholipid ratio. Our data show that the rate of transformation was dependent on the amount of HDL added and was more rapid at 37 °C than at 0 °C. The formation of an apoprotein–vesicle complex necessitates dissociation of apoprotein from the HDL particle. Dissociation of apoprotein from HDL was reported to occur in vitro (Scanu & Granda, 1966; Nichols et al., 1976) and calorimetric studies indicated apoA-1 was loosely associated with HDL particles and can be readily dissociated by heating (Tall et al., 1977).

The transformed product of the interaction between intact HDL and vesicles was similar in size to HDL on the basis of the elution profile on Sepharose 4B. EM studies also indicated that vesicles were broken down into smaller particles. As noted previously, a discoidal particle with a size and density similar to HDL was characterized as a product of the interaction between isolated apoproteins and a variety of phospholipid preparations. Similar particles may have been formed in the present study. It is possible that a portion of the vesicle-associated lipid was incorporated into, or attached onto, intact HDL particles. Small changes in the size of HDL would not be distinguished by gel chromatography since particles as different in size as HDL_2 and HDL_3 were not clearly resolved by chromatographic techniques.

The cholesteryl oleate incorporated into EYL vesicles underwent transfer more rapidly than lecithin. Although we have not ruled out cholesterol ester exchange as the cause of this phenomenon, cholesteryl ester exchange does not occur readily between lipoproteins in vitro (Goodman & Lequire, 1975). The physical properties of cholesteryl ester containing vesicles have not been elucidated, and it is conceivable that two populations of identically sized vesicles may exist in the sonicated preparations, one population containing more labeled cholesteryl ester than the other, and having properties which impart a greater tendency to interact with HDL and undergo transformation. A more likely explanation would be that cholesteryl ester molecules are dispersed equally throughout a homogeneous vesicle population, but tend to cluster in one region of the vesicle bilayer. The phospholipid–cholesteryl ester complex in such a region may be less stable than surrounding regions of lecithin and, therefore, more susceptible to disruption by apoproteins. Accumulation of cholesteryl ester within a phospholipid bilayer was recently suggested to occur during the metabolism of nascent HDL particles (Hamilton et al., 1976).

The ability of intact HDL to interact with artificial phospholipid bilayers containing either cholesterol or cholesteryl esters may have physiological relevance. HDL has been implicated in the process of cellular cholesterol efflux (Glomset & Norum, 1973), and apoprotein–phospholipid complexes have been shown to remove cholesterol and phospholipid from cultured cells (Jackson et al., 1975; Stein et al., 1976). In addition, the catabolism of triglyceride-rich lipoproteins is probably associated with transfer of phospholipid into plasma HDL (Havel, 1957). During lipolysis of chylomicrons in vitro the surface lipids form folds of bilayer membrane (Blanchette-Mackie & Scow, 1976). It is likely that chylomicron or VLDL surface lipids are transferred into HDL by mechanisms analogous to the interactions of EYL vesicles with HDL.

References

Assman, G., & Brewer, H. B., Jr. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 989.

Atkinson, D., Smith, H. M., Dickinson, J., & Austin, J. P. (1976) *Eur. J. Biochem. 64*, 541.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Blanchette-Mackie, E. J., & Scow, R. O. (1976) *J. Lipid Res. 17*, 57.

Brecher, P., Chobanian, J., Small, D. M., & Chobanian, A. V. (1976) *J. Lipid Res. 17*, 239.

Brecher, P. I., Pyun, H. Y., & Chobanian, A. V. (1977) *J. Lipid Res. 18*, 154.

Cham, B. E., & Knowles, B. R. (1976) *J. Lipid Res. 17*, 176.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497.

Forte, T., Nichols, A. V., Gong, E. L., Lux, S., & Levy, R. I. (1971) *Biochim. Biophys. Acta 248*, 381.

Glomset, J. A., & Norum, K. R. (1973) *Adv. Lipid Res. 11*, 1.

Goodman, A. D., & Lequire, V. S. (1975) *Biochim. Biophys. Acta 398*, 325.

Gregoriadis, G. (1976) *N. Engl. J. Med. 295*, 704.

Gregoriadis, G., & Neerunjun, D. E. (1974) *Eur. J. Biochem. 47*, 179.

Hamilton, R. L., Williams, M. C., Fielding, C. J., & Havel, R. J. (1976) *J. Clin. Invest. 58*, 667.

Havel, R. J. (1957) *J. Clin. Invest. 36*, 848.

Havel, R. J., Kane, J. P., & Kashyap, M. L. (1973) *J. Clin. Invest. 52*, 32.

Huang, C. (1969) *Biochemistry 8*, 344.

Jackson, R. L., Gotto, A. M., Stein, O., & Stein, Y. (1975) *J. Biol. Chem. 250*, 7204.

Juliano, R. L., & Stamp, D. (1975) *Biochem. Biophys. Res. Commun. 63*, 651.

Krupp, L., Chobanian, A. V., & Brecher, P. I. (1976) *Biochem. Biophys. Res. Commun. 72*, 1251.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

McDougall, I. R., Dunnick, J. K., McNamee, M. G., & Kriss, J. P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3487.
Morrisett, J. D., Gallagher, J. G., Aune, K. C., & Gotto, A. M., Jr. (1974) *Biochemistry 13*, 4771.
Nichols, A. V., Gong, E. L., Blanche, P. J., Forte, T. M., & Anderson, D. W. (1976) *Biochim. Biophys. Acta 446*, 226.
Novosad, A., Knapp, R. D., Gotto, A. M., Pownall, H. J., & Morrisett, J. D. (1976) *Biochemistry 15*, 3176.
Pittman, R. C., Khoo, J. C., & Steinberg, D. (1975) *J. Biol. Chem. 250*, 4505.
Rudel, L. L., & Morris, M. D. (1973) *J. Lipid Res. 14*, 364.
Sata, T., Havel, R. J., Kotite, L., & Kane, J. P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1063.
Scanu, A., & Granda, J. L. (1966) *Biochemistry 5*, 446.
Scanu, A., & Wisdom, C. (1972) *Annu. Rev. Biochem. 41*, 703.
Scanu, A., Toth, J., Edelstein, C., Koga, S., & Stiller, E. (1969) *Biochemistry 8*, 3309.
Stein, O., Vanderhoek, J., & Stein, Y. (1976) *Biochim. Biophys. Acta 431*, 347.
Tall, A. R., & Small, D. M. (1977) *Nature (London) 265*, 163.
Tall, A. R., Deckelbaum, R. J., Small, D. M., & Shipley, G. G. (1977) *Biochim. Biophys. Acta 487*, 145.
Tyrell, D. A., Heath, T. D., Colley, C. M., & Ryman, B. F. (1976) *Biochim. Biophys. Acta 457*, 259.

Alteration of Ribosomal Protein L6 in Gentamicin-Resistant Strains of *Escherichia coli*. Effects on Fidelity of Protein Synthesis†

Robert Kühberger, Wolfgang Piepersberg, Angelika Petzet, Peter Buckel,‡ and August Böck*

ABSTRACT: The effect of alterations in ribosomal protein L6 of gentamicin-resistant mutants of *Escherichia coli* on in vitro and in vivo polypeptide synthesis was studied. It was found that mutant ribosomes possess altered misreading properties by the following criteria. (1) Poly(U)-dependent incorporation of isoleucine or of a mixture of amino acids in the presence of misreading-inducing aminoglycosides like streptomycin, neomycin, or gentamicin is reduced in extracts from the mutants; this reduction of the misreading is a property of the 50S and not of the 30S subunit or the 100000*g* supernatant fraction. (2) Incorporation of L-histidine into the (normally histidine-less) coat protein from phage R17 or into a protein electrophoretically comigrating with the coat protein is stimulated by gentamicin in the case of wild-type but not with mutant ribosomes. Unlike streptomycin, gentamicin affects R17 RNA dependent polypeptide synthesis by wild-type ribosomes in a multiphasic way which supports the notion of multiple binding sites for the drug. Synthesis on mutant ribosomes on the other hand is monophasicly inhibited. The size classes of translation products made in the presence of gentamicin by wild-type ribosomes are very different from those obtained without antibiotic. (3) The leaky translation of the *argF40* amber codon [Rosset, R., & Gorini, L. (1969) *J. Mol. Biol. 39*, 95] is prevented or reduced by mutations in protein L6, and low concentrations of gentamicin or streptomycin antagonize this restriction, rendering the strains conditionally drug dependent. The different response of ribosomes with L6 or S12 (*strA*) mutations to aminoglycosides suggests that restriction of ribosomal ambiguity by these alterations occurs by different mechanisms and that the L6 alteration changes a parameter of the ribosome function which is involved in the codon recognition process.

In order to obtain information on the mode of action of the gentamicin group of aminoglycoside antibiotics we have previously undertaken an analysis of strains of *Escherichia coli* resistant to gentamicin sulfate (Buckel et al., 1977). It was found that mutants which are moderately resistant to this antibiotic possess at least two mutations which act cooperatively to express resistance. Whereas one class of these mutations interferes with the uptake of the drug by the bacteria (our unpublished results), the other one affects ribosomal properties (Buckel et al., 1977). For several strains an alteration of the 50S subunit protein L6 could be demonstrated. The L6 alteration confers an increase in the level of resistance to all misreading-inducing aminoglycosides, and its action is apparently independent from that of the classical mutations in the *strA* gene (structural gene for ribosomal protein S12) which determine resistance to streptomycin (Strigini & Gorini, 1970; Funatsu & Wittmann, 1972; Wittmann & Wittmann-Liebold, 1974).

The present study deals with the response to gentamicin in vitro of ribosomes from the L6 mutants. Evidence is presented which shows that these mutations can restrict translational ambiguity.

Materials and Methods

Bacterial Strains and Growth Conditions. Escherichia coli strains GE20-8, GS20-10, and GS50-15 are gentamicin-resistant derivatives (Buckel et al., 1977) of the parental strain A19 (thi⁻ met⁻ *rna* λ⁺) (Gesteland, 1966). They possess a mutationally altered ribosomal protein, L6, in addition to a second mutation interfering with gentamicin uptake (Buckel et al., 1977; our unpublished results). Strains TD3 and TD6 were constructed from mutant GS20-10 by P1 transduction; TD3 possesses the wild-type form and TD6 the mutant form of L6. In addition, both contain a streptomycin resistance mutation in ribosomal protein S12 (Buckel et al., 1977). Bacteria were grown at 37 °C either in 250-mL batches in 1-L Erlenmeyer flasks on rotatory incubators or in 10-L

† From the Lehrstuhl für Mikrobiologie der Universität Regensburg, Regensburg, West Germany. *Received May 30, 1978.* This work was supported by a grant from the Deutsche Forschungsgemeinschaft.
‡ Present address: Boehringer Mannheim GmbH, Biochemica Werk, Tutzing, West Germany.

0006-2960/79/0418-0187$01.00/0