Biochimica et Biophysica Acta, 556 (1979) 369—387

BBA 78495

INTERACTION OF PLASMA APOLIPOPROTEINS WITH LIPID MONOLAYERS

R.L. JACKSON [a], F. PATTUS [b,*] and R.A. DEMEL [b,**]

[a] *Department of Pharmacology and Cell Biophysics, Biological Chemistry and Medicine, University of Cincinnati College of Medicine, Cincinnati, OH (U.S.A.) and* [b] *The Biochemical Laboratory, State University of Utrecht, Padualaan 8, Utrecht (The Netherlands)*

(Received January 10th, 1979)

Key words: Lipid monolayer; Plasma lipoprotein; Lipid-protein interaction; Lipolysis

Summary

The monolayer technique has been used to study the interaction of lipids with plasma apolipoproteins. Apolipoprotein C-II and C-III from human very low density lipoproteins, apolipoprotein A-I from human high density lipoproteins and arginine-rich protein from swine very low density lipoproteins were studied. The injection of each apoprotein underneath a monolayer of egg phosphatidyl[^{14}C]choline at 20 mN/m caused an increase in surface pressure to approximately 30 mN/m. With apolipoprotein C-II and apolipoprotein C-III there was a decrease in surface radioactivity indicating that the apoproteins were removing phospholipid from the interface; the removal of phospholipid was specific for apolipoprotein C-II and apolipoprotein C-III. Although there was a removal of phospholipid from the monolayer, the surface pressure remained constant and was due to the accumulation of apoprotein at the interface. The rate of surface radioactivity decrease was a function of protein concentration, required lipid in a fluid state and, of the lipids tested, was specific for phosphatidylcholine. Cholesterol and phosphatidylinositol were not removed from the interface. The addition of 33 mol% cholesterol to the phosphatidylcholine monolayer did not affect the removal of phospholipid by apolipoprotein C-III.

The addition of phospholipid liposomes to the subphase greatly facilitated the apolipoprotein C-II-mediated removal of phospholipid from the interface.

* Present address: Universität Bern, Freiestrasse 1, Bern, Switzerland.

** To whom reprint requests should be sent.

Abbreviations: chylomicrons, $d < 0.95$ g/cm^3; VLDL, very low density lipoproteins ($d = 0.95$—1.006 g/cm^3); LDL, low density lipoproteins ($d = 1.006$—1.063 g/cm^3); HDL, high density lipoproteins ($d = 1.063$—1.210 g/cm^3). The A-B-C nomenclature of Alaupovic [1] is used to designate the apoproteins. In addition, apo E or 'arginine-rich' protein has been used.

Although apolipoprotein A-I and arginine-rich protein gave surface pressure increases, phospholipid was only slightly removed from the interface by the addition of liposomes. Based on these findings, we conclude that the apolipoproteins C interact specifically with phosphatidylcholine at the interface. This interaction is important as it relates to the transfer of the apolipoproteins C and phospholipids from very low density lipoproteins to other plasma lipoproteins.

The addition of human plasma high density lipoproteins or very low density lipoproteins to the subphase increased the apolipoprotein C-mediated removal of phosphatidyl[^{14}C]choline from the interface 3—4 fold. Low density lipoproteins did not affect the rate of decrease. During lipolysis of very low density lipoproteins to the subphase increased the apolipoprotein C-mediated removal of with the lipid monolayer. Lipolysis experiments were performed in a monolayer trough containing a surface film of egg phosphatidyl[^{14}C]choline and a subphase of very low density lipoproteins and bovine serum albumin. Lipolysis was initiated by the addition of purified milk lipoprotein lipase to the subphase. As a result of lipolysis, there was a decrease in surface radioactivity of phosphatidylcholine. The pre-addition of high density lipoproteins decreased the rate of decrease in surface radioactivity. From these results, we suggest that high density lipoproteins function as acceptor for the products of lipolysis and are of importance as it relates to the metabolic relationship between very low density lipoproteins and high density lipoproteins in man.

Introduction

Chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) are the major carriers of lipid in blood (for reviews, Refs. 2—4). Each lipoprotein class contains specific proteins or apoproteins. Because of their lipid-binding properties, the plasma apolipoproteins have been used extensively as model proteins to study lipid-protein interaction (for review, Refs. 5—7). In these studies, purified apoproteins have been added to phospholipid vesicles and it has generally been shown that there are changes in the physical state of the proteins and the lipids. By these bulk systems it has been difficult to determine any differences in the apoproteins for their affinity to lipids.

In the present investigation, we have utilized monolayer techniques to study the lipid-protein interaction. Four representative plasma apolipoproteins have been used in these studies and include apolipoprotein C-II and C-III (apo C-II and apo C-III) from human VLDL, apo A-I from human HDL and swine arginine-rich protein (apo E), an apoprotein found in normal human lipoproteins but in increased quantities in animals fed cholesterol-rich diets [8]. It will be shown that only the apo C proteins interact with phospholipid monolayers and cause removal of lipid from the interface. If phospholipid liposomes were then added to the subphase, the decrease was greatly facilitated. The relevance of this interaction to the transfer of apolipoproteins and phospholipids during catabolism of triacylglycerol-rich lipoproteins is considered also.

It is well-established that human plasma very low density lipoproteins (VLDL) are degraded in the capillary endothelium by lipoprotein lipase (for review, see Ref. 9). With hydrolysis of triacylglycerol, there is a loss of the surface components (cholesterol, phospholipids and proteins) from the lipoprotein particle. The proteins, mainly the apo C proteins, are then found associated with the high density lipoproteins (HDL). The mechanism for the transfer of the apo C proteins from VLDL to HDL is not known. It is also not clear whether the apoproteins are transferred from the lipoprotein interface alone or with a complement of phospholipid and cholesterol. Since plasma lipoproteins are the acceptor of the apo C protein during lipoprotein metabolism, we have expanded the monolayer studies and have shown that plasma VLDL and HDL also interact with apoprotein-phospholipid complexes to facilitate their removal from interfaces. In addition, we have utilized monolayer techniques to investigate the effects of lipolysis on VLDL and the mechanism of transfer of the apo C proteins. From these results we conclude that HDL serve as an acceptor of the apo C proteins during lipolysis.

Materials and Methods

Isolation of lipoproteins and apoproteins. Apo C-II and apo C-III were isolated from VLDL which were obtained from plasma of fasting subjects with types IV and V hyperlipoproteinemia as described previously [10]. The VLDL were isolated by ultracentrifugation flotation of plasma in a 60 Ti rotor (Beckman Inst.) for 20 h at 58 000 rev./min and 8°C. After delipidation with diethyl ether/ethanol (3 : 1) and then diethyl ether, 8 ml of 0.01 M Tris-HCl, pH 7.6, containing 3 M guanidine HCl was added to the lipid-free VLDL (250 mg). After stirring for 1 h at 25°C, the insoluble material was removed by centrifugation at 20 000 rev./min. The soluble protein material (150 mg) was applied to a column (2.6 × 160 cm) of Sephracryl S-200 (Pharmacia) equilibrated with 0.01 M Tris-HCl, pH 7.6, containing 3 M guanidine HCl; the column was eluted with the same equilibration buffer. Those fractions containing the apo C proteins were combined and desalted on Bio Gel P-2 in 0.1 M ammonium bicarbonate. The apo C proteins were further fractionated on DEAE-cellulose (Whatman DE-52 cellulose) at 4°C. The column (1.6 × 24 cm) was equilibrated with 0.01 M Tris-HCl, pH 8.2, containing 6 M urea. The equilibration and elution buffers were prepared at the time of chromatography and were made from urea which had been passed through a column of Rexyn I-300 immediately before use. The proteins were eluted with a linear gradient which consisted of 500 ml of the equilibration buffer on one side of the gradient maker and 500 ml of the same buffer containing 0.125 M NaCl on the other side. The fractions corresponding to apo C-II and apo C-III-1 (apo C-III containing one residue of sialic acid) were pooled and desalted at 4°C by chromatography on Bio Gel P-2 in 0.1 M ammonium bicarbonate. The purity of the isolated proteins was determined by analytical isoelectrofocusing between pH 4.0 and 6.0 [11] and by amino acid analysis.

Human apo A-1 was prepared from HDL as described previously [12]. The isolated protein was homogenous by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) and by amino acid analysis.

Swine arginine-rich protein was isolated from VLDL and β-VLDL which

were obtained from plasma of cholesterol-fed Hampshire barrow pigs. The swine were fed a diet consisting of a stock hog chow [13] to which was added 15% lard and 2% cholesterol (by weight); the animals were fed the diet 3 months prior to bleeding. Plasma was collected as described previously [13]. VLDL was isolated by ultracentrifugal flotation of plasma in KBr at $d \cdot 1.020$. After delipidation and solubilization in 0.01 M Tris-HCl, pH 7.6, containing 3 M guanidine HCl as described above for apo C proteins, arginine-rich protein was isolated by chromatography of the soluble protein on Sephacryl S-200. The conditions of chromatography were exactly the same as above. Individual fractions were analyzed by polyacrylamide gel electrophoresis in SDS, those fractions containing apo E were pooled, desalted on Bio Gel P-2 in 0.1 M ammonium bicarbonate and lyophilized. The amino acid composition of the isolated protein was consistent with that reported by Kahley et al. [14].

Apoprotein concentrations were determined by amino acid analysis. Each of the purified apoproteins contained less than 0.02 mol% phospholipid as determined by the method of Bartlett [15]. Apo C-II was iodinated by the method of Roholt and Pressman [16]. Sodium[^{125}I]iodide (Amersham) was added to a stock solution of 3 mM iodine/9 mM potassium iodide to give $4.86 \cdot 10^4$ cpm/mol iodide. Iodination of apo C-II (1 mg) was performed in 1 ml of 0.05 M Tris-HCl, pH 9.6, by the addition of 1 mol equivalent of iodine. After 30 min incubation at 0°C, the apoprotein was dialyzed extensively against 0.05 M Tris-HCl, pH 7.6, with several changes of buffer. The modified protein contained 0.43 mol iodide with $1.88 \cdot 10^7$ cpm/mg apo C-II. ^{125}I-labelled apo C-II was incorporated into VLDL by the following procedure. To VLDL (1.2 mg protein) was added ^{125}I-labelled apo C-II (0.56 mg) in a final volume of 5.0 ml of 0.10 M Tris-HCl, pH 7.6. After 6 h at 23°C, the ^{125}I-labelled VLDL were isolated by ultracentrifugal flotation at 49 000 rev./min for 18 h at 8°C the ^{125}I-labelled apo C-II-labelled VLDL contained $2.50 \cdot 10^6$ cpm/mg protein.

Preparation of lipids. Phosphatidylcholine was isolated from egg yolk and purified with silicic acid chromatography. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (dipalmitoyl phosphatidylcholine) was synthesized as described previously [17]. [^{14}C]Methyl groups were introduced into egg phosphatidylcholine according to the method of Stoffel et al. [18] using [^{14}C]methyl iodide (Amersham); the specific activities of the lipids were 40 and 45 Ci $\cdot$ mol^{-1}, respectively. Phosphatidyl[^{14}C]inositol (specific activity 1 Ci $\cdot$ mol^{-1}), was prepared as before [19]. Lysophospholipids were absent in all phospholipid preparations as determined by thin-layer chromatography. Cholesterol and 26-[^{14}C]cholesterol (Amersham, 58 Ci $\cdot$ mol^{-1}) were purified by thin-layer chromatography before use.

In vitro lipolysis experimental procedures. Lipoprotein lipase was purified from bovine milk by affinity chromatography on heparin-Sepharose as described by Kinnunen [20]. The purified enzyme had a specific activity of 36 nmol fatty acids released/h per mg protein using glycerol [^{14}C]triolein emulsified in the presence of 5% gum arabic as substrate [21]. As determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the purified enzyme was homogenous with a molecular weight of approximately 55 000. The enzyme was stored in 50% glycerol/0.05 M Tris-HCl, pH 7.6, at —20°C and added directly to the incubation mixture.

Interfacial measurements. Monolayer studies were performed in a 15 ml Teflon trough (5.4 × 5.9 × 0.5 cm). A 1.5 × 1.5 cm extended corner with a hole of 0.8 cm was made for the injection of protein underneath the monolayer. The trough was filled with 0.05 M Tris-HCl, pH 7.4. The subphase was stirred with a magnetic bar. The surface pressure was determined with a recording Beckman LM 500 electrobalance with a platinum plate 1.96 cm wide. The surface radioactivity was measured with a gas flow detector (Nuclear Chicago 8731) 0.3 cm above the interface. The gas used was helium/1.3% butane, the window, micromil 150 $\mu g/cm^2$, 4.2 × 1.3 cm.

^{125}I-labelled proteins were collected from the interface in a counting vial and counted in a packard auto-spectrometer. Corrections were made for the amount of subphase collected.

Lipid films were spread from a chloroform/10% methanol solution until the desired interfacial pressure was reached. Apoproteins were dissolved in 0.05 M Tris-HCl, pH 7.6, containing 6 M guanidine HCl to give 1 mg/ml and were incubated at 22°C for 2 h prior to injection underneath the monolayer. Unless stated otherwise, all measurements were performed at 37°C in a thermostatically controlled box filled with nitrogen [19].

Other methods. Phospholipid phosphorus was determined by the method of Bartlett [15] ^{125}I radioactivity was determined in a Packard auto-scintillation spectrometer. Liposomes containing egg phosphatidylcholine and 2 mol% egg phosphatidic acid were prepared by sonication of a hand-shaken dispersion until the suspension was clear. Apoprotein concentrations were determined by amino acid analysis. Lipoprotein protein concentrations were determined by the method of Schacterle and Pollack [22]. Triglycerides were quantitated by autoanalyzer techniques [23]. Fatty acids were extracted from the lipolysis incubation mixture using the extraction procedure of Dole [24] and were quantitated by gas chromatography [25].

Results

Interaction of apo C-II with phosphatidylcholine monolayers

In the absence of a monolayer, apo C-II collected at the air-water interface to a final pressure of 18.2 mN/m. Therefore, most of the experiments performed in these studies were done at an initial pressure of the lipid monolayer of 20 mN/m.

The injection of apo C-II underneath a monolayer of egg phosphatidyl[^{14}C]-choline at 20 mN/m caused an increase in surface pressure of 8—10 mN/m (Fig. 1A); the time for maximal changes in surface pressure was inversely proportional to the amount of apo C-II added to the subphase. However, regardless of the initial surface pressure (20—30 mN/m) or the amount of apoprotein added (4—50 μg), the final surface pressure was approximately 30 mN/m. As seen in Fig. 1A, after the maximal attainment of surface pressure, there was a decrease in surface radioactivity, indicating a loss of radiolabelled phosphatidylcholine from the interface to the subphase. At an initial surface pressure of 20 mN/m, the rate of surface radioactivity decrease was proportional to the amount of apo C-II injected into the subphase (Fig. 2) and was maximal with 3 nmol apoprotein. Of particular significance in Fig. 1A was the

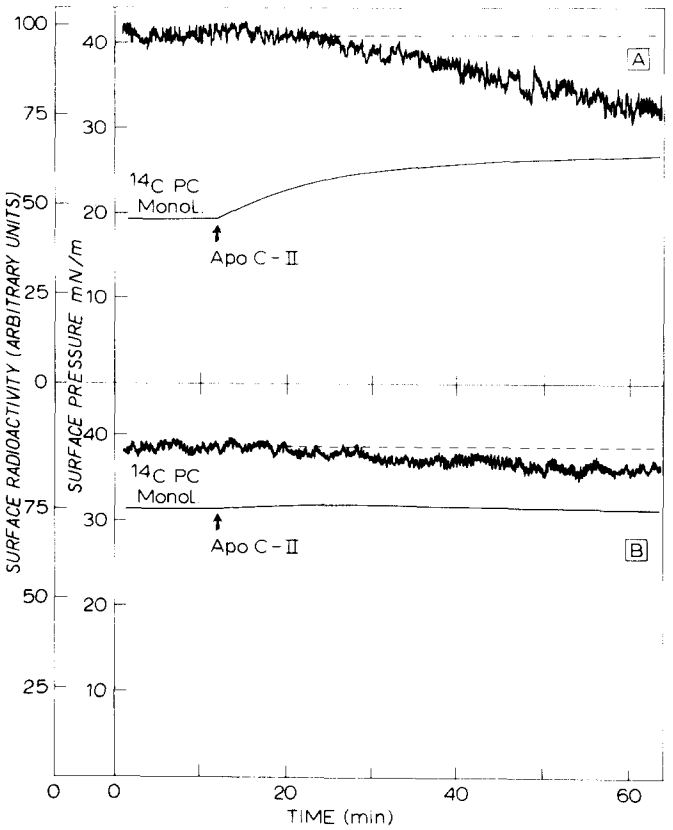

Fig. 1. (A) Interaction of apo C-II with egg phosphatidylcholine (PC) monolayer. The monolayer consisted of 6 nmol egg phosphatidyl[*Me*-^{14}C]choline at an initial pressure 20 mN/m. The subphase contained 15 ml of 0.05 M Tris-HCl, pH 7.4. At the indicated time, apo C-II (25 μg) was added to the subphase. (B) The same experimental conditions as (A), except that the initial surface pressure was 30 mN/m.

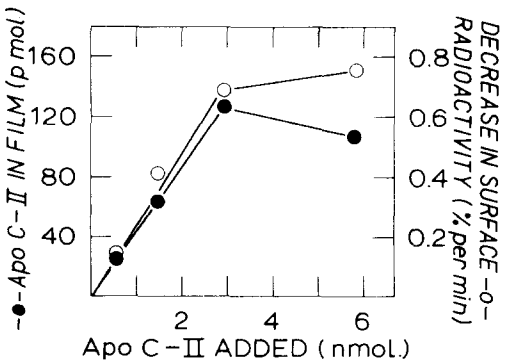

Fig. 2. Effects of apo C-II on egg phosphatidylcholine (PC) monolayer. The experimental conditions were the same as those described in the legend to Fig. 1A. To the subphase was added the indicated amount of ^{125}I-labelled apo C-II ($2.09 \cdot 10^4$ cpm/nmol). After 1 h, the film was collected and ^{125}I radioactivity determined; the equivalent volume was removed from the subphase, counted and substracted from the values found for the film. The decrease in surface radioactivity was determined by drawing a line through the linear portion of the curve.

finding that although the surface radioactivity was decreasing, the surface pressure remained nearly constant suggesting that apo C-II was replacing the phospholipid which was leaving the interface. To test this possibility, ^{125}I-labelled apo C-II was utilized. In these experiments ^{125}I-labelled apo C-II gave the same rate of surface radioactivity decrease as unmodified apo C-II (0.23% · min^{-1} · $nmol^{-1}$ apo C-II injected). As shown in Fig. 2, the increase in surface radioactivity decrease is accompanied by a corresponding increase in ^{125}I-labelled apo C-II at the interface, thus maintaining a constant surface pressure. When 3 nmol ^{125}I-labelled apo C-II were added to the subphase, approximately 120 pmol apo C-II collected at the interface after 1 h, or 4% of the added protein. The decrease of the surface radioactivity was 42% corresponding to 2.5 nmol phospholipid. When the subphase was removed and phospholipid radioactivity determined, 2.5 nmol of the phosphatidyl[^{14}C]choline was present in the subphase. This result suggests that the decrease in surface radioactivity was due to the removal of phospholipid from the interface. When the phosphatidyl[^{14}C]choline present in the subphase was added to either human plasma VLDL or to HDL, 20—30% of phospholipid radioactivity was present at $d < 1.006$ and $d = 1.063$—1.210, respectively.

Lipid packing is of critical importance for removal of phospholipid from the monolayer. As shown in Fig. 1B, when apo C-II is injected underneath a monolayer of egg phosphatidylcholine at an initial surface pressure of 30 mN/m, there was only a small increase of surface pressure. Although the final pressure is nearly the same as that in Fig. 1A, the decrease in surface radioactivity was negligible.

To test the possibility that the physical state of the phospholipid is important for the removal of lipid from the interface, we utilized dipalmitoyl phosphatidylcholine, a phospholipid which has a thermotropic phase transition at approximately 29 mN/m at 37°C. As seen in Fig. 3, the injection of apo C-II underneath a dipalmitoyl phosphatidylcholine monolayer at 37°C and 22 mN/m gave an increase in surface pressure of 6 mN/m. However, with these conditions there was no decrease in surface radioactivity. When the temperature was increased to 44°C and the initial surface decreased to 15 mN/m (conditions giving rise to decreased lipid packing), there was a final increase in surface pressure to 20 mN/m and the radioactivity decreased with a rate of 0.30% · min^{-1} · $nmol^{-1}$ apo C-II injected. At 15 mN/m the phospholipid was presumably in a physical state such that the apoprotein could interact with the phospholipid and remove it from the interface. If the temperatures were lowered to 15°C at 15 mN/m, then there was no decrease in surface radioactivity (Fig. 3C); the phospholipid was again in a crystalline state.

Interaction of apo C-II with other lipids

The increase in surface pressure observed by adding apo C-II to the subphase of a lipid monolayer was not specific for phosphatidylcholines. Fig. 4 illustrates the surface pressure increase using cholesterol or phosphatidylinositol. Similar increases were found (data not shown) with 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol, 1,2-diethermyristoyl-*sn*-glycero-3-phosphatidylcholine and ergosterol. Although not all lipids were tested, it can be seen in Fig. 4 that apo C-II did not cause a decrease

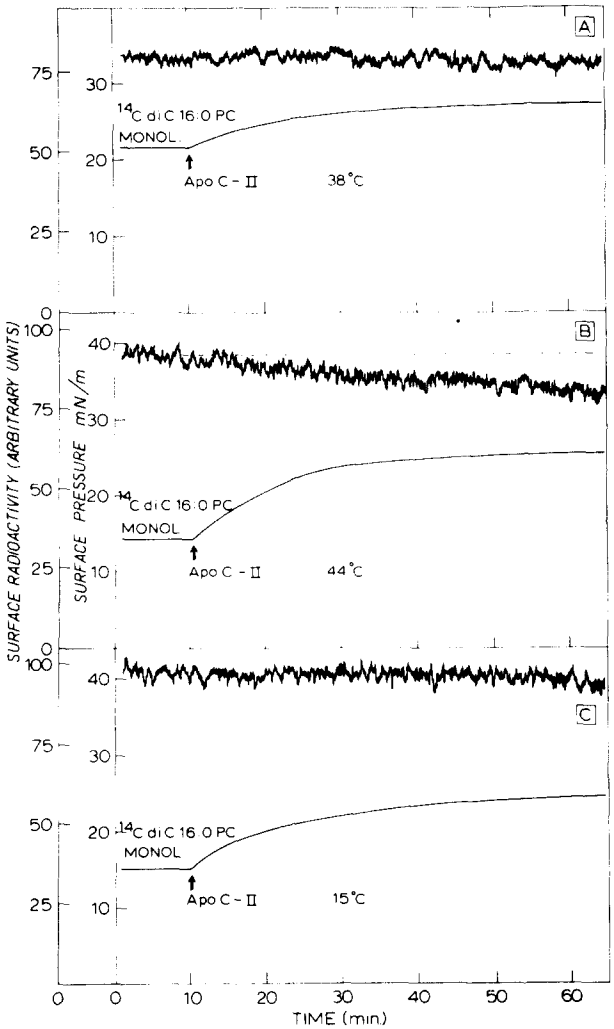

Fig. 3. (A) Effects of phospholipid structure on the interaction of apo C-II with dipalmitoyl phosphatidylcholine (di C 16 : 0 PC). The monolayer consisted of 6 nmol dipalmitoyl phosphatidyl[*Me*-^{14}C]choline at 20 mN/m and 38°C. At the indicated time, apo C-II (25 μg) was added to the subphase. (B) The experimental conditions were identical to (A), except the initial surface pressure was 15 mN/m and the temperature 44°C. (C) The experimental conditions were identical to (A), except the initial surface pressure was 15 mN/m and 15°C.

in surface radioactivity using monolayers of [^{14}C]cholesterol or phosphatidyl-[^{14}C]inositol, suggesting that the interaction of apo C-II was specific for phosphatidylcholine.

Interaction of other proteins with phospholipid monolayers and the addition of liposomes to the subphase

To test the specificity of the lipid-protein interaction with respect to the protein moiety, we also used apo C-III, apo A-I, arginine-rich protein and fatty acid-free bovine serum albinum and phospholipid monolayers. When each of

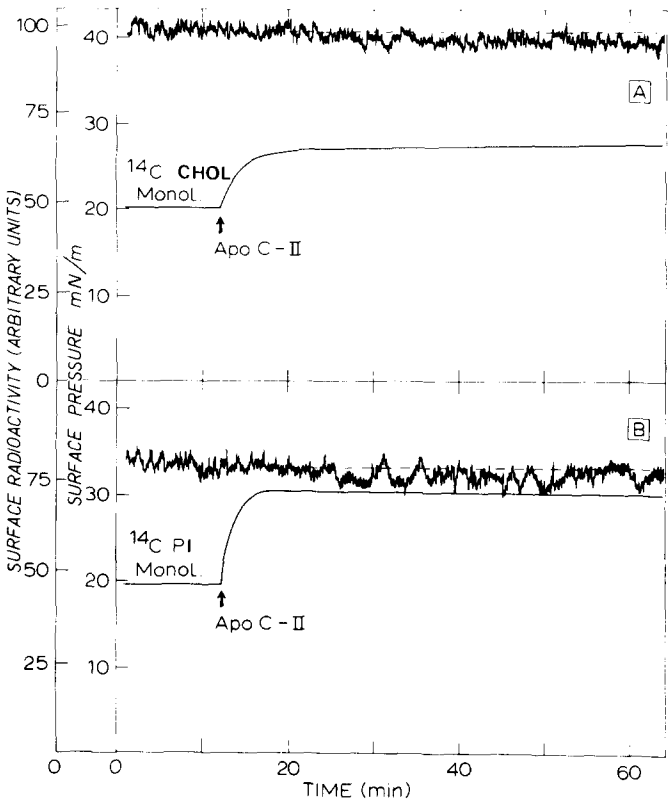

Fig. 4. Effects of apo C-II on cholesterol (Chol) and phosphatidylinositol (PI) monolayers. (A) The monolayer consisted of 9 nmol [^{14}C]cholesterol at 20 mN/m. At the indicated time, apo C-II (25 μg) was injected into the subphase. (B) The monolayer consisted of 6 nmol phosphatidyl[^{14}C]inositol at an initial pressure of 20 mN/m. At the indicated time, apo C-II (25 μg) was injected into the subphase.

these proteins was injected underneath a monolayer of egg phosphatidylcholine at 20 mN/m, there was an increase in surface pressure of 6—10 mN/m for the apoproteins and 2 mN/m for albumin (Fig. 5). However, only with apo C-III there was a significant decrease in surface radioactivity. Using ^{125}I-labelled apo A-I and arginine-rich protein, it was found that 1—2% of the apoprotein added to the subphase collected at the interface. Nonetheless, these apoproteins did not remove phosphatidylcholine from the interface.

Since previous studies [5—7] have shown that the apo C proteins interact with vesicles of phospholipid, it was of interest to determine if the addition of liposomes to the subphase affected the rate of phospholipid removed from the interface. As shown in Fig. 5A the addition of liposomes to a subphase, to which apo C-III had been previously added, greatly facilitated the removal of phosphatidylcholine from the interface. There was also a small decrease in surface pressure. The addition of liposomes to a subphase containing arginine-rich protein also facilitated the decrease in surface radioactivity. However, the addition of liposomes had no effect on surface radioactivity or pressure when either apo A-I or bovine serum albumin was used.

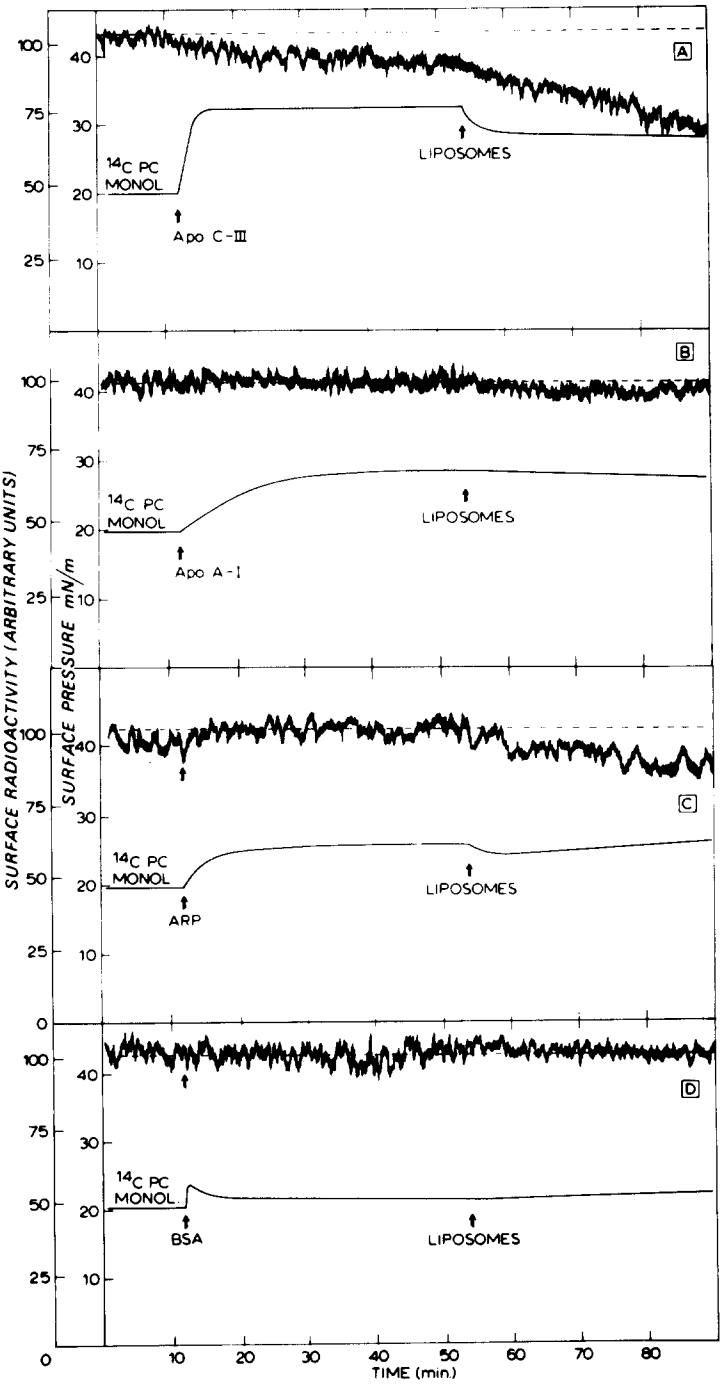

Fig. 5. Effects of apolipoproteins and fatty acid-free bovine serum albumin on egg phosphatidylcholine (PC) monolayers. In each experiment the monolayer consisted of 6 nmol egg phosphatidyl[*Me*-^{14}C]-choline at an initial pressure of 20 mN/m. At the indicated times the proteins were added to the subphase. (A) Apo C-III (25 μg). (B) Apo A-I (25 μg). (C) Arginine-rich protein (apo E) (25 μg). (D) Bovine serum albumin (BSA) (25 μg). At the indicated time, 1 μmol liposomal egg phosphatidylcholine containing 2 mol% phosphatidic acid was added to the subphase.

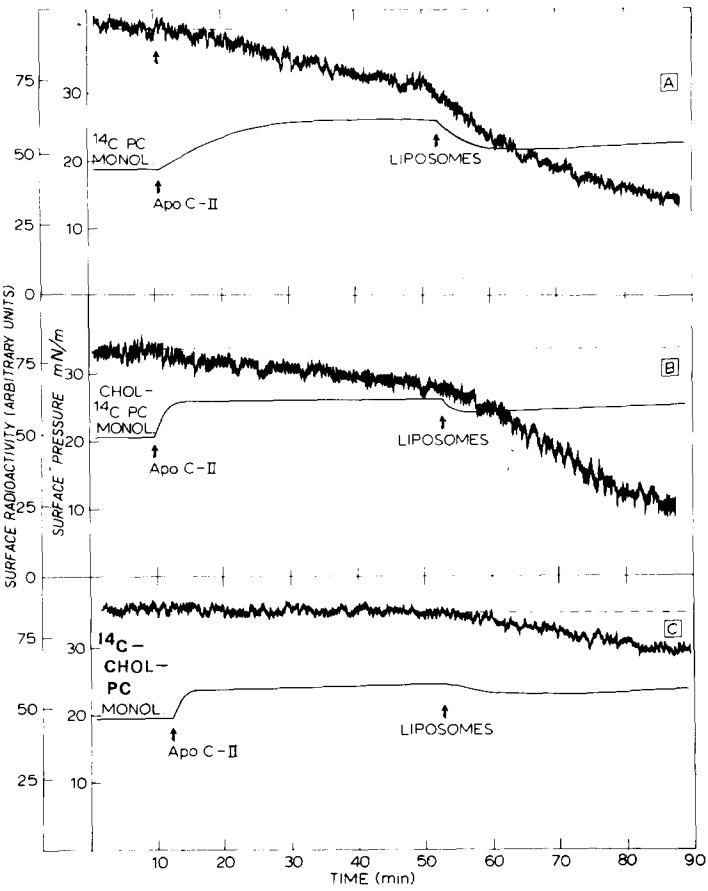

Fig. 6. Effects of apo C-II on mixed monolayers of egg phosphatidylcholine (PC) and cholesterol (Chol). (A) The monolayer consisted of 6 nmol egg phosphatidyl[*Me*-^{14}C]choline at an initial surface pressure of 20 mN/m. At the indicated time, apo C-II (25 μg) was added to the subphase. After 1 h, 1 μmol liposomal egg phosphatidylcholine containing 2 mol% phosphatidic acid was added to the subphase. (B) The experimental conditions were identical to (A), except the monolayer contained 3.6 nmol egg phosphatidyl-[*Me*-^{14}C]choline and 3.6 nmol cholesterol. (C) The experimental conditions were identical to (A) except the monolayer contained 3.6 nmol egg phosphatidylcholine and 3.6 nmol [^{14}C]cholesterol.

Effects of apo C-II on mixed monolayers

Since it is well-established that the degradation of VLDL by lipoprotein lipase [26] is accompanied by removal of cholesterol, phospholipid and apo C proteins, it was of interest to utilize a mixed monolayer of cholesterol and phospholipid. As seen in Fig. 6, the addition of 33 mol% cholesterol to an egg phosphatidyl[^{14}C]choline monolayer did not affect the rate by which apo C-II decreased the surface radioactivity. When radioactive cholesterol was used there was no decrease in surface radioactivity after the addition of apo C-II indicating that cholesterol is not removed from the interface. However, the removal of phosphatidylcholine from a mixed film of phosphatidylcholine/[^{14}C]cholesterol, after the subsequent addition of apo C-II and liposomes (Fig. 6B) is accompanied with some removal of cholesterol (Fig. 6C).

Effects of serum lipoproteins on apo C-induced reduction of surface radioactivity

Since HDL are the physiologic acceptors for the apo C proteins during lipolysis [9], it was of interest to determine the effects of HDL on the rate of decrease of surface radioactivity. As shown in Fig. 7A, the injection of HDL (1.0 mg protein, 0.8 μmol phospholipid) underneath a monolayer of egg phosphatidyl[^{14}C]choline caused an increase in the rate of decrease of surface radioactivity by apo C-II; injection of HDL alone without apo C-II caused no decrease in surface radioactivity (shown in Fig. 8). However, since 1 mg HDL contains approximately 10% [9] or 100 μg apo C proteins, and since the apo C proteins exchange between VLDL and HDL, it was possible that the increased

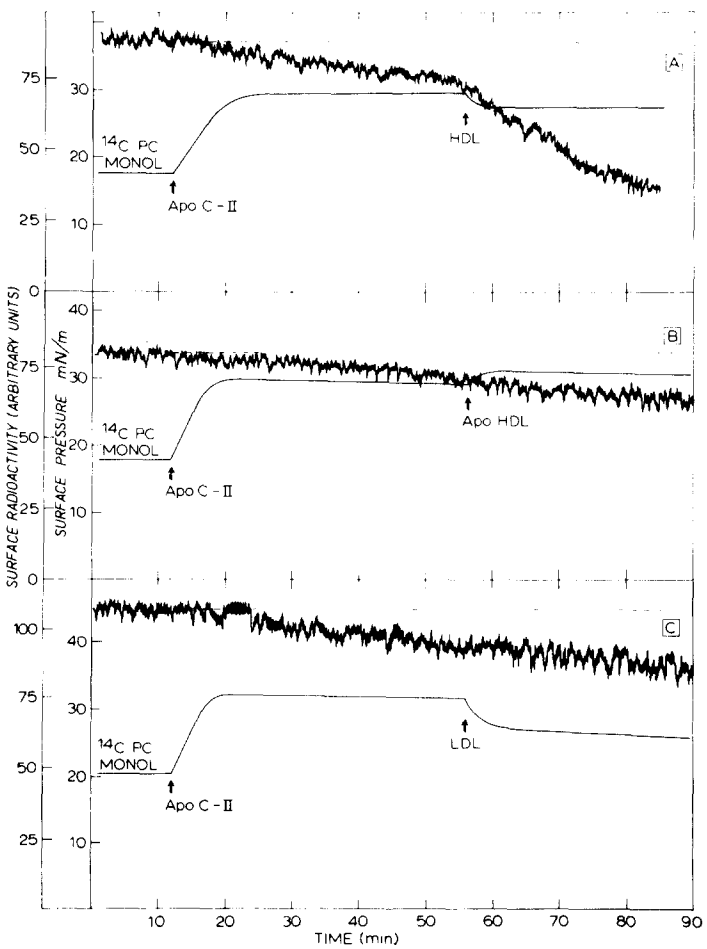

Fig. 7. Effects of human plasma lipoproteins on the interaction of apo C-II with egg phosphatidylcholine (PC) monolayers. As indicated, apo C-II (25 μg) in 0.05 ml 0.10 M Tris-HCl, 6 M guanidine HCl, pH 7.4, was injected under a monolayer of egg phosphatidyl[^{14}C]choline (PC), 6 nmol at 20 mN/m. After 1 h lipoproteins or apolipoproteins were then injected under the monolayer. (A) High density lipoproteins (HDL, d = 1.063—1.210) 1.0 mg protein, 0.8 μmol phospholipid. (B) Lipid-free HDL (apo HDL) 1.0 mg. (C) Low density lipoproteins (LDL, d = 1.020—1.050) 1.0 mg protein, 1.5 μmol phospholipid. For details of the monolayer equipment and experimental details refer to text.

rate (Fig. 7A) was the result of the additional apoprotein to the trough. To investigate this possibility, HDL were delipidated and the lipid-free proteins (apo HDL) were added to the subphase. As seen in Fig. 7B, the injection of apo HDL (1 mg) did not affect the rate of decrease of surface radioactivity. Thus, we conclude from the results shown in Fig. 7A and B that HDL interact with apo C-II in a monolayer of egg phosphatidyl[^{14}C]choline and enhance the rate of apoprotein-phospholipid removal from the interface.

To further determine the specificity of the interaction between lipoproteins and apoprotein-phospholipid interfaces, LDL was injected under the monolayer (Fig. 7C). It is known that LDL are derived from the lipolysis of VLDL and contain virtually no apo C proteins [9]. As seen in Fig. 7C injection of LDL (1.0 μmol phospholipid) underneath a monolayer of egg phosphatidyl-[^{14}C]choline to which apo C-II had been previously added did not enhance the rate of decrease of surface radioactivity. In addition to HDL, injection of VLDL under a monolayer of egg phosphatidyl[^{14}C]choline to which apo C-III had been previously added also caused an enhanced rate of decrease of surface radioactivity.

As a prerequisite to study the release of apo C from VLDL, a number of experiments were necessary to establish the monolayer system. The experiments were performed in a 15 ml Teflon monolayer trough at 37°C, on which was spread a monolayer of either egg phosphatidyl[^{14}C]choline or didecanoyl phosphatidylcholine. The reagents were then injected underneath the monolayer and changes in surface pressure and surface radioactivity were observed. To bind the fatty acids produced during lipolysis, it was necesary to add fatty acid-free bovine serum albumin to the incubation mixture. In separate experiments, it was determined that the addition of bovine serum albumin to a final concentration of 5 mg/ml (75 mg total) was the optimal concentration required to bind fatty acids produced during lipolysis. The injection of bovine serum albumin under an egg phosphatidyl[^{14}C]choline monolayer at an initial surface pressure of 20 mN/m caused only a slight increase in surface pressure. At this surface pressure, bovine serum albumin had no effect on the surface radioactivity. Furthermore, the addition of VLDL did not effect surface radioactivity.

It was also necessary to establish that milk lipoprotein lipase does not hydrolyze the phospholipid monolayer. It is known [27—29] that lipoprotein lipase has phospholipase activity and hydrolyzes 80—90% of chylomicron and VLDL phospholipids during lipolysis. However, as with many lipolytic enzymes [30], lipoprotein lipases do not penetrate a phospholipid interface at high (greater than 20 mN/m) surface pressure to cause hydrolysis of the lipid. The injection of lipoprotein lipase (1.5 μg) underneath a monolayer of egg phosphatidyl[^{14}C]choline at 20 mN/m did not cause a decrease in surface radioactivity. We have utilized a short-chained phospholipid, didecanoyl phosphatidylcholine at an initial surface pressure of 20 mN/m. The addition of lipoprotein lipase to the subphase did not cause a decrease in surface pressure. The decrease in surface radioactivity induced by the addition of apo C-III was not changed by the presence of lipoprotein lipase.

As additional proof that lipoprotein lipase does not hydrolyze phospholipid monolayers, we have extracted the subphase and found no lysophosphatidylcholine.

Fig. 8A shows the changes in surface pressure and radioactivity of a phospholipid film when lipoprotein lipase is added to a subphase containing VLDL. After the injection of the lipase, there was an increase in surface pressure and then after a lag of 5—10 min a decrease in surface radioactivity. An increase in surface pressure was also found when just the apoproteins were added to the trough. However, with bovine serum albumin present in the system, the magnitude of the increase was not as great. The rate of decrease of surface radioactivity was significantly greater than that observed by the apoproteins alone (Fig. 7) and was similar to that observed when HDL were added to the subphase after apo C-III and phospholipid interaction (Fig. 7A). What the acceptor is for the apoprotein-phospholipid complex is unclear but may be a VLDL remnant which is formed during lipolysis [9].

To show that the products of lipolysis are responsible for the decrease in surface radioactivity, HDL were added to the subphase. As shown in Fig. 8B, the addition of increasing amounts of HDL to the subphase caused a decrease in the rate of surface radioactivity leaving the interface.

In the next series of experiments, we have attempted to determine the fate of apo C-II during lipolysis. As shown above apo C-II interacts with egg phos-

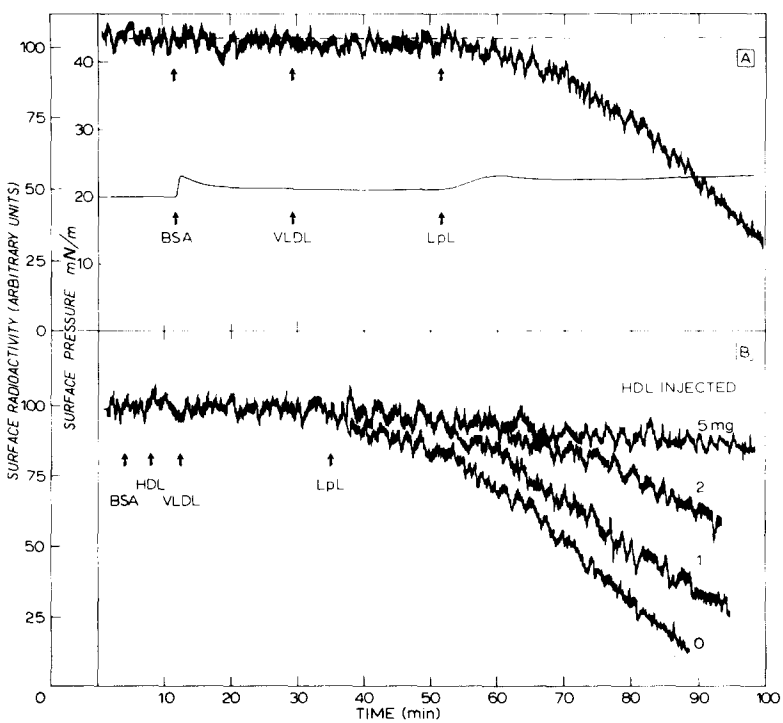

Fig. 8. Effects of lipolysis on monolayers of egg phosphatidylcholine (PC). In each panel, the monolayer consisted of egg phosphatidyl[^{14}C]choline (PC, 6 nmol) at an initial surface pressure of 20 mN/m. As indicated fatty acid-free bovine serum albumin (BSA, 75 mg in 0.75 ml 0.01 M Tris, pH 7.4), human plasma very low density lipoproteins (VLDL, 85 μg protein), 1.0 μmol triacylglycerol, human plasma high density lipoproteins (HDL) at the indicated amount and bovine milk lipoprotein lipase (LpL, 1.5 μg in 0.025 ml 0.05 M Tris-HCl, 50% glycerol, pH 7.6) was injected under the monolayer.

TABLE I

FATE OF ^{125}I-LABELLED APO C-II DURING IN VITRO LIPOLYSIS OF VERY LOW DENSITY LIPOPROTEINS

The experiment was carried out in a 15 ml Teflon trough containing a monolayer of didecanoyl phosphatidylcholine at an initial surface pressure of 20 mN/m. The subphase contained 15 ml of fatty acid-free bovine serum albumin (5 mg/ml) in 0.05 M Tris-HCl, pH 7.4. ^{125}I-labelled-VLDL was prepared by incorporating ^{125}I-labelled-apo C-II ($1.88 \cdot 10^7$ cpm/mg) into VLDL as described in Materials and Methods. To the subphase was added as indicated ^{125}I-labelled-VLDL or unlabelled VLDL (0.3 mg proteins, 1.0 μmol triglyceride), high density lipoproteins (HDL, 5 mg), low density lipoproteins (LDL, 5 mg) bovine milk lipoprotein lipase (1.5 μg) and apo C-II (labelled or unlabelled), 50 μg. After 1 h at 37°C, the film was collected and the percent apo C-II at the interface determined.

Experiment	Additions		Lipoprotein lipase (μg)	Percent ^{125}I-labelled apo C-II in film
	Apoprotein	Lipoprotein		
1	^{125}I-labelled apo C-II	—	—	6.25
2	^{125}I-labelled apo C-II	VLDL	—	1.50
3	—	^{125}I-labelled VLDL	—	0.73
4	apo C-II	^{125}I-labelled VLDL	—	2.00
5	—	^{125}I-labelled VLDL	1.5	3.42
6	—	^{125}I-labelled VLDL + HDL	1.5	0.70
7	—	^{125}I-labelled VLDL + HDL	1.5	1.93

phatidyl[^{14}C]choline monolayers to cause a decrease in surface radioactivity. With the decrease in radioactivity there was an increase in the amount of apo C-II that accumulated at the interface, thus maintaining constant surface pressure. Table I shows that when the system contained ^{125}I-labelled apo C-II alone 6.25% of the apoprotein was at the interface after 1 h. If unlabelled VLDL were added to the subphase and then ^{125}I-labelled apo C-II added, the percentage of apo C-II at the interface decreased to 1.5%. This decrease is probably a result of exchange of ^{125}I-labelled apo C-II for unlabelled apo C-II in the VLDL and as a result unlabelled apo C-II is available to interact with the phospholipid monolayer. If the ^{125}I-labelled apo C-II is incorporated into VLDL and then the labelled VLDL is added to the subphase, 0.73% of the apo C-II was at the interface. The addition of unlabelled apo C-II to ^{125}I-labelled VLDL increased the amount of apo C-II at the interface to 2.0%. Thus, these experiments show that apo C-II exchanges between VLDL particles. During lipolysis, apo C-II is released and if there is no acceptor for the apoprotein, it is free to interact with the surface monolayer. As shown in Table I, 3.42% of the apo C-II in VLDL was collected at the interface after lipolysis. In this experiment there was no acceptor for the apo C-II. However, when HDL were added to the subphase in addition to VLDL, only 0.70% of the apo C-II was at the interface; ^{125}I-labelled apo C-II which was released during lipolysis bound to HDL giving less apo C-II to interact with the interface. As shown in Fig. 7C, LDL did not effect the rate of decrease of surface radioactivity. The addition of LDL to the subphase (Table I) had some effect on the amount of apo C-II at the interface. However, the amount was three times greater than that found for HDL.

The fate of ^{125}I-labelled apo C-II during lipolysis was also determined by ultracentrifugation. Using ^{125}I-labelled VLDL alone without any acceptor for apo C-II, 18% of the apo C-II was isolated at $d < 1.063$ and 53% at $d > 1.210$. When HDL was added to the subphase the majority of the apo C-II was isolated between $d = 1.063$—1.210 (75%); only 2% of the apoprotein was isolated at $d < 1.063$. Thus, HDL serve as an acceptor for the apo C-II which is released during lipolysis. Finally, we have fractionated the HDL into HDL_2 ($d = 1.063$—1.120) and HDL_3 ($d = 1.120$—1.210). HDL_2 and HDL_3 contained 24 and 76%, respectively, of the total ^{125}I-labelled apo C-II recovered in the HDL. Furthermore, the specific activity of each fraction was nearly identical.

Discussion

In the absence of a lipid monolayer, all of the apoproteins studied were surface active and collected at the air/water interface to a final surface pressure of approximately 18 mN/m. When apo A-I, apo C-II, apo C-III, or argine-rich protein were injected underneath a lipid monolayer which was at an initial surface pressure of 20 mN/m, all the apoproteins caused an increase in surface pressure to approximately 30 mN/m. Between lipid surface pressures of 20 and 30 mN/m, the final surface pressure attained after injection of the protein was independent of the initial surface pressure, the type of lipid at the interface or, within limits, the amount of protein added to the subphase. However, the time required to reach 30 mN/m was dependent on the final protein concentration. Since all of the apoprotein tested showed increase in surface pressure that was independent of the type of lipid at the interface, it seems unlikely that the increase in surface pressure was due to a specific interaction between the apoproteins and lipids. A more likely explanation is that the apoproteins penetrate the interface in a nonspecific manner causing increased packing of the lipid molecules. There is, however, specificity with respect to the type of lipid removed from the interface and the proteins which facilitated the removal. Of the lipids tested, only phosphatidylcholines were removed from the interface whereas expanded phospholipid monolayers were required. A significant finding in this investigation was that only apo C-II and apo C-III caused a decrease in the surface radioactivity of phosphatidylcholine. Human apo C-II and apo C-III were also obtained from Dr. P.H.E. de Groot (University of Rotterdam); both of these preparations also showed a decrease in surface radioactivity.

The findings in this investigation that apo C-II and apo C-III interact with phospholipid monolayers to cause removal of lipid from the interface whereas apo A-I and arginine-rich protein do not demonstrate a clear difference in the lipid-binding properties of the plasma apolipoproteins. Although we cannot rule out the possibility, it seems unlikely that these differences can be explained by protein aggregation since all of the proteins were first incubated with 6 M guanidine HCl prior to injection underneath the monolayer. The rate of removal of lipid from the interface by apo C-II and apo C-III was increased when liposomes were added to the subphase. The nature of the complex formed between apo C-II and apo C-III and phospholipid is unknown.

A discussion of the complex formed between apo C-II and phospholipid is pertinent as it relates to lipoprotein metabolism. The apo C proteins and

possibly phospholipids can be transferred in vivo and in vitro between VLDL and HDL [26,32]. The distribution of the apo C proteins between VLDL and HDL in vivo is determined primarily by the plasma triglyceride concentration. Kashyap et al. [33] found that when plasma triglyceride levels are greater than 500 mg/100 ml nearly all of the apo C-II is associated with the triacylglycerol-rich lipoproteins. During lipolysis the apo C proteins return to HDL. It is not clear as to whether apoproteins are transferred to HDL with a complement of phospholipid. Studies by Illingworth and Portman [34] and Eisenberg [35] indicate that exchange of apo C proteins between VLDL and HDL occurs independently of that of the bulk of phospholipid. On the other hand, the dat data of Rubenstein and Rubenstein [36] suggest that the transfer of apo C occurs as an apoprotein-phospholipid complex. In the study of Eisenberg [35], the exchange of apo C proteins and phospholipid was a temperature-dependent process. At 37°C, apo C exchange occurred within a few minutes, whereas phospholipid exchange required 60 min. However, it could not be ruled out that some phospholipids could be transferred with the apoprotein.

The data presented in the present report suggest that the apo C proteins can interact with phosphatidylcholines. The physiological significance of this finding is related to the metabolic role of apo C. It is known that during lipolysis there is a loss of apo C, phospholipid and cholesterol from the surface monolayer of VLDL and that HDL are the acceptors of the products of lipolysis in vivo. In the present in vitro study of lipolysis using purified milk lipoprotein lipase, we have shown that the release of apo C-II from VLDL during lipolysis was not dependent on the addition of plasma or HDL to the incubation mixture.

In the absence of HDL, there was a rapid removal of phospholipid from the lipid monolayer after lipolysis of VLDL by lipoprotein lipase in the subphase (Fig. 8A). The amount of ^{125}I-labelled apo C-II in the film was five-fold greater in the absence of HDL compared to its present (Table I, Expts. 5 and 6) whereas the pre-addition of increasing amounts of HDL to the subphase reduced the rate by which phospholipid was removed (Fig. 8B). When the incubation mixture contained VLDL and HDL at a protein ratio (w/w) of 5 (ratio found in plasma of normal fasting subjects), the rate of decrease of surface radioactivity was negligible.

Fig. 9 gives a schematic representation of the release of apo C from VLDL after lipolysis by lipoprotein lipase. (a) The interaction of the released apo C with phosphatidylcholine at the interphase and the binding of the apo C-phosphatidylcholine complex by HDL, which was added after lipolysis (compare Fig. 7A). (b) The direct interaction of the released apo C with HDL, which was added before lipolysis (compare Fig. 8B). The importance of apo C-II transfer from HDL and VLDL and vice versa is undoubtedly related to the activator properties of apo C-II; adipose and muscle lipoprotein lipase require apo C-II for maximal activity. The present study and that of Patsch et al. [31] underline that HDL can serve as the acceptor of apo C during lipolysis. This conclusion is different from that of Alaupovic [1] who have proposed that the HDL density range contains discrete lipoproteins families designated LpA and LpC.

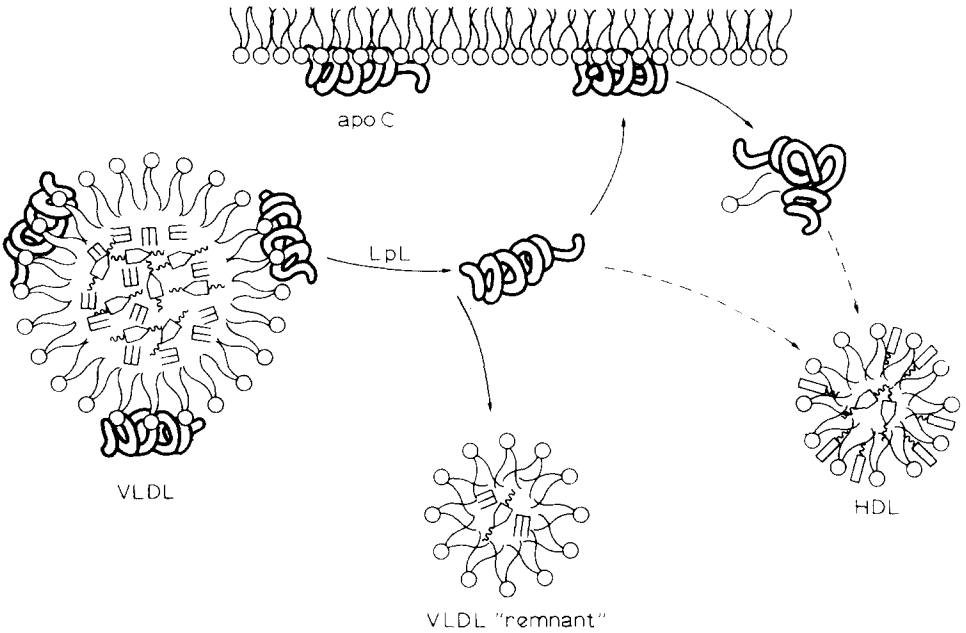

Fig. 9. Fate of apo C proteins during lipolysis.

Acknowledgements

These studies were performed during the stay of R.L.J. as visiting professor at the State University of Utrecht. The author wishes to thank Professor Gerard de Haas for arranging the visit and to him and Professor Laurens van Deenen for their helpful discussions. Special thanks go to Dr. Fred A. Kummerow, Burnsides Research Laboratory, University of Illinois, Urbana, IL, U.S.A., for supplying the swine plasma for these studies and Dr. P.H.E. de Groot, Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands, for a sample of apolipoprotein C-II and C-III. This work was supported by U.S. Public Health Service grants HL-22619 and 23019; by the American Heart Association; by the Lipid Research Clinic Program of the National Heart, Lung and Blood Institute (NIH NHLI 72-2914); and by General Clinical Research Center grant RR-00068.

References

1 Alaupovic, P. (1971) Atherosclerosis 12, 141—146
2 Scanu, A.M., Edelstein, C. and Keim, P. (1975) in The Plasma Proteins: Structure, Function and Genetic Control (Putman, F.W., ed.), Vol. I, 2nd edn., pp. 317—391, Academic Press, New York
3 Jackson, R.L., Morrisett, J.P. and Gotto, A.M. (1976) Physiol. Rev. 56, 259—316
4 Kane, J.P. (1977) in Lipid Metabolism in Mammals (Synder, F., ed.), Vol. I, pp. 209—257, Plenum Press, New York
5 Gennis, R.B. and Jonas, A. (1977) Annu. Rev. Biophys. Bioeng. 6, 195—238
6 Osborne, J.C. and Brewer, H.B. (1977) Adv. Protein Chem. 31, 253—337
7 Morrisett, J.D., Jackson, R.L. and Gotto, A.M. (1977) Biochim. Biophys. Acta 472, 93—133

8 Mahley, R.W. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M. and Ontko, J.A., eds.), pp. 181—197, The American Physiology Society, Bethesda, MD
9 Schaefer, E.J., Eisenberg, S. and Levy, R.I. (1978) J. Lipid Res. 19, 667—687
10 Jackson, R.L., Baker, H.N., Gilliam, E.B. and Gotto, A.M. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1942—1945
11 Catapano, A.L., Jackson, R.L., Gilliam, E.B., Gotto, A.M. and Smith, L.C. (1978) J. Lipid Res. 19, 1047—1052
12 Baker, H.N., Jackson, R.L. and Gotto, A.M. (1973) Biochemistry 12, 3866—3871
13 Jackson, R.L., Morrisett, J.D., Pownall, H.J., Gotto, A.M., Kamio, A., Imai, H., Tracy, R. and Kummerow, F.A. (1977) J. Lipid Res. 18, 182—190
14 Mahley, R.W., Weisgraber, K.H., Innerarity, T., Brewer, H.B. and Assmann, G. (1975) Biochemistry 14, 2817—2823
15 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
16 Roholt, O.A. and Pressman, D. (1972) Methods Enzymol. 25, 438—448
17 Van Deenen, L.L.M. and de Haas, G.H. (1964) Adv. Lipid Res. 2, 167—234
18 Stoffel, W., Lekim, D. and Tschung, T.S. (1971) Hoppe-Seyler's Z. Physiol. Chem. 352, 1058—1064
19 Demel, R.A., Kalsbeek, R., Wirtz, K.W.A. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 466, 10—22
20 Kinnunen, P.K.J. (1977) Med. Biol. 55, 187—191
21 Ehnholm, C., Kinnunen, P.K.J. Huttunen, J.K.,Nikkilä, E.A. and Ohta, M. (1975) Biochem. J. 149, 649—655
22 Schacterle, G.R. and Pollack, R.L. (1973) Anal. Biochem. 51, 654—655
23 Manual of Laboratory Methods (1972) Lipid Research Clinics Program, University of North Carolina, Chapel Hill, NC
24 Dole, V.P. (1965) J. Clin. Invest. 35, 150—154
25 Mac Gee, J. and Allen, K.G. (1974) J. Chromatogr. 100, 35—41
26 Glangeaud, M.C., Eisenberg, S. and Olivecrona, T. (1977) Biochim. Biophys. Acta 586, 23—35
27 Scow, R.O. and Egelrud, T. (1976) Biochim. Biophys. Acta 431, 538—549
28 Ehnholm, C., Shaw, W., Greten, H. and Brown, W.V. (1975) J. Biol. Chem. 250, 6756—6761
29 Eisenberg, S. (1977) Biochim. Biophys. Acta 489, 337—342
30 Demel, R.A., Geurts, van Kessel, W.S.M., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 97—107
31 Patsch, J.R., Gotto, A.M., Olivecrona, T. and Eisenberg, S. (1978) Proc. Natl. Acad. Sci. U.S. 75, 4519—4523
32 Eisenberg, S. (1976) in Atherosclerosis Review (Paoletti, R. and Gotto, A.M., eds.), Vol. I, pp. 23—60, Raven Press, New York
33 Kashyap, M.L., Srivastava, L.S., Chen, C.Y., Perisutti, G., Campbell, M., Lutmer, R.F. and Glueck, C.J. (1977) J. Clin. Invest. 60, 171—180
34 Illingworth, D.R. and Portman, O.W. (1972) Biochim. Biophys. Acta 280, 281—289
35 Eisenberg, S. (1978) J. Lipid Res. 19, 229—236
36 Rubenstein, B. and Rubenstein, D. (1972) J. Lipid Res. 13, 317—324