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Research Article

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INTRODUCTION

An arginine-rich apoprotein (ARP)[1] initially described by Shore and Shore (1) in human very low density lipo-

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[1] *Abbreviations used in this paper*: ARP, arginine-rich apoprotein; SDS, sodium dodecyl sulphate; VLDL, very low density lipoprotein.

protein (VLDL) was purified for chemical characterization using guanidine-Sepharose chromatography (2). The molecular size and some of the chemistry of the apoprotein was determined, but because of the complete insolubility of the apoprotein when guanidine was removed, investigations of its function were limited. Although in humans this apoprotein is predominantly associated with the triglyceride-rich lipoproteins, no role for it in plasma-triglyceride metabolism has been described. It has been noted that the apoprotein occurs in VLDL with high contents of cholesterol ester (3), leading to the suggestion that it is related to plasma sterol ester transport. Studies of lecithin-cholesterol acyltransferase-deficient patients (4, 5) have demonstrated increments of ARP in triglyceride-rich lipoproteins when active lecithin-cholesterol acyltransferase is added to the system, supporting a role for the apoprotein in plasma cholesterol ester metabolism.

The present study reports the observation that the ARP either free in solution, or on triglyceride-rich lipoproteins, binds to heparin and can be isolated on a heparin affinity column. The binding occurs in the absence of divalent cations. This binding also permits the separation of the bulk of triglyceride-rich lipoproteins which contain ARP and bind to heparin from a small amount of triglyceride-rich lipoprotein in the plasma which contains virtually no ARP and are eluted in the unbound fraction. Derivatization of the apoprotein suggests that the arginine residues on this apoprotein have an important role in heparin binding.

METHODS

Apoprotein preparation. Blood was collected from normal humans and patients with elevated plasma VLDL concentrations (type IV phenotype) in disodium EDTA (1 mg/ml). All subjects were sampled after a 14-h fast. The plasma was separated from the erythrocytes by centrifugation at 2,500 rpm for 20 min in a Sorvall model RC 2-B refrigerated centrifuge (Du Pont Co., Instrument Products Div., Wilmington, Del.) at 8°C. Lipoproteins were isolated by a modification of the method described by Havel et al. (6). Chylomicrons were removed from the plasma of each subject by ultracentrifugation at the native plasma density for 25 min at 20,000 rpm (1 × 10⁶ g-min) in a Spinco model L-2 centrifuge using a 40-rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto,

Calif.). VLDL (S_f 20–400) were isolated from the chylomicron-free plasma by ultracentrifugation at 40,000 rpm for 18 h (1 × 10⁸ g-min) at density 1.006 at 8°C. VLDL were floated through saline twice by a similar ultracentrifugation and were dialyzed against 200 vol of 0.01% EDTA buffered to pH 7.4 before delipidation. The VLDL were delipidated at 4°C by three extractions with ethanol-diethyl ether (3:1) using 50 vol of solvent per volume of lipoprotein. The resulting apoprotein contained <0.2% lipid as phospholipid.

Affinity chromatography. A heparin affinity column was prepared by covalently linking heparin to cyanogen bromide-activated Sepharose 4B and subsequently reacting the mixture with ethanolamine exactly as described by Kato and Anfinsen (7) and Iverius (8). [³⁵S]Heparin (The Radiochemical Centre, Amersham, England) was included in some of the preparations to determine the amount of heparin bound to the gel. Other chemicals and radiochemicals were obtained from commercial sources. A mean of 0.4–0.5 mg heparin/ml wet gel was observed in these preparations. The column was made to a height of 12 cm with a diameter of 2.5 cm, and equilibrated with 100 ml of 0.12 M NaCl, 2 mM sodium phosphate, pH 7.4. The apoproteins previously prepared were extracted several times with 5-ml aliquots of 0.12 M NaCl, 2 mM phosphate, pH 7.4, to an apoprotein concentration of 0.5–2.5 mg/ml. The buffer-soluble apoproteins, in a 20–30-ml volume, were applied to the affinity column at a rate of ≅30 ml/h. The column was eluted at a similar rate with 400 ml of a linear salt gradient from 0.05 to 1.5 M NaCl in 2 mM phosphate, pH 7.4, and the eluates were monitored at 280 nm.

After the buffer-soluble apoproteins were obtained, the insoluble residue was reextracted with 5 M urea 2 mM sodium phosphate (pH 7.4) 0.05 M NaCl buffer. The urea-soluble apoproteins were applied to the heparin affinity column that had been equilibrated with the urea buffer and then run in a 0.05–0.5 M NaCl gradient in the urea phosphate system. The urea-insoluble residue was lyophilized and dissolved in the soidum dodecyl sulfate (SDS) buffer for subsequent electrophoresis to estimate the amount of insoluble ARP. Native whole VLDL were also applied to the column at protein concentrations of 0.5–1.0 mg/ml in 10–30 ml and run under the same conditions as the buffer-soluble apoproteins. The mean recovery of protein from the column was >90% for both whole lipoproteins and apoproteins.

Polyacrylamide electrophoresis. The procedure used was that of Weber and Osborn (9). Gels of 10% acrylamide were prepared in a 0.1 M phosphate buffer, pH 7.0, and 0.2% SDS. Samples of ≅45 μg of protein were mixed with one drop of glycerol, 5 μl of mercaptoethanol, 50 μl of buffer, and 3 μl of tracking dye. The samples, along with protein standards of known molecular weight, were applied to the gel and subjected to electrophoresis at 360 V for 2–5 h in 0.05 M phosphate (0.1% SDS) buffer, pH 7.0. The gels were stained with either Coomassie Blue or amido-Schwartz and were destained with a solution containing 7.5% acetic acid and 5% methanol in water at 60°C with changes of solution every half-hour until completion. The gels were scanned using a Gilford gel scanner (Gilford Instruments Laboratories Inc., Oberlin, Ohio) and the responses integrated by planimetry. Isoelectric focus was run in a Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.) using Ampholines (LKB-Produkter, Bromma, Sweden) in either the pH 3–10 or 4–6 range according to an established method (10).

Amino acid analysis. The analyses were carried out according to the procedure of Spackman et al. (11) on a Beckman model 121 analyzer (Beckman Instruments Inc., Fullerton, Calif.). Protein samples dialyzed free of NaCl were routinely hydrolyzed for 24 h with oxygen-free 6 N HCl at 108°C in a nitrogen atmosphere. Hydrolysates were dried in a vacuum dessicator over sodium hydroxide, dissolved in 0.005 N HCl, and applied to the analyzer column. NH₂-terminal analysis

was performed on the heparin affinity isolated apoprotein by the dansyl method exactly as previously described (2).

Protein analysis and modification. Protein determinations were performed according to the method of Lowry et al. (12) using bovine albumin as a standard. Both the isolated apoprotein from the affinity column and native VLDL were derivatized with phenylglyoxal exactly as described by Takahashi for bovine RNAse (13). The phenylglyoxal was dialyzed away from the apoprotein or lipoprotein in 1,000 vol of appropriate buffer for subsequent incubation or chromatography. No change in the SDS-polyacrylamide electrophoretic behavior or immunochemical reactivity was noted for the ARP after phenylglyoxal treatment.

Lipoprotein incubations. Freshly prepared VLDL were incubated in a 0.03 M Tris, pH 7.4, 0.12 M NaCl buffer at concentrations of 2 mg protein/ml and 0.03 μCi [³⁵S]heparin (CalAtomic, Inc., Los Angeles, Calif.) (sp act 0.2 μCi/mg). The incubations were performed at 37°C for 3 h in a Dubnoff metabolic shaker. The lipoprotein was isolated by ultracentrifugation and delipidated as previously described. The incubation buffer was added to the apoproteins to obtain a soluble apoprotein concentration of ≅10 mg/ml and this solution containing [³⁵S]heparin was used for immunochemical evaluations. Native and phenylglyoxal-derivatized VLDL at protein concentrations from 1–5 mg/ml were incubated with 0.03 μCi/ml [³⁵S]heparin, sp act 0.2 μCi/mg in a 0.1 M N-ethylmorpholine buffer at pH 5.5 for 3 h at 37°C. The lipoproteins were isolated by ultracentrifugation and along with the infranate, assayed for radioactivity. The incubation buffer was changed to more acid conditions to stabilize the VLDL derivative (13). No differences were observed for heparin binding to fresh VLDL in this buffer as compared to the tris buffer at higher pH.

Immunodiffusion. Antisera to apoproteins were placed in the center well of a standard immunodiffusion system (14), and the aqueous-soluble apoproteins from VLDL which had been incubated with [³⁵S]heparin and concentrated in a UM-2 Amicon Filter (Amicon Corp., Lexington, Mass.) were placed in peripheral wells. A blank well with the same content of [³⁵S]heparin was included in each immunodiffusion study. After development in a humid chamber of 24–48 h, the immunoprecipitin bands were cut from the gels and placed in counting vials, ground to small particle size with a pestle, and dispersed in 10 ml of Aquasol for radioactivity assay in a Packard model 3375 Liquid Scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The antisera to the individual C apolipoproteins² and the ARP were prepared in our laboratory by sequential immunizations of rabbits. Other antisera were obtained from commercial sources.

Lipid analysis. Triglyceride determinations on the lipoprotein fractions were obtained by an automatic fluorometric technique (15). Cholesterol and cholesterol esters were assayed by the Schoenheimer-Sperry method (16). Phospholipid determinations were performed on the extracts by Folch et al. (17) of the lipoproteins by the method of Ames and Dubin (18).

RESULTS

After ethanol ether delipidation, most of the VLDL apoproteins soluble in phosphate buffer were the previously characterized C apolipoproteins (19, 20). A group of uncharacterized apoproteins having molecular

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² C apolipoproteins, these include the apo-Ser, apo-Glu, and apo-Ala proteins named according to the carboxy terminus.
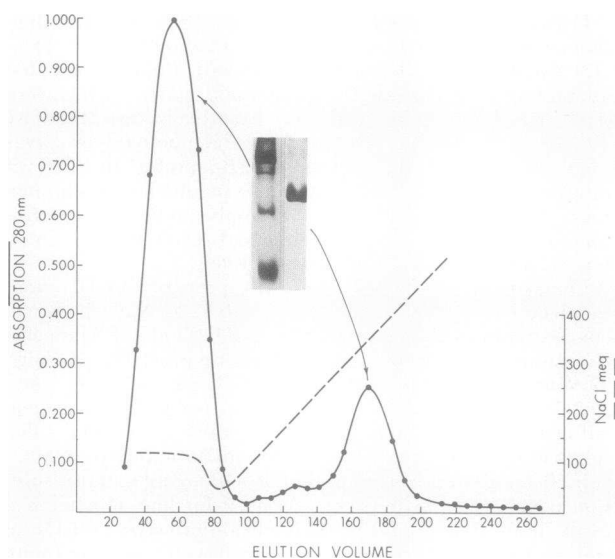
FIGURE 1 Heparin affinity chromatography of 26 mg of soluble VLDL apoprotein. This was applied to the column in 25 ml of buffered 0.12 M NaCl solution and eluted with a salt gradient from 0.05 to 1.5 M NaCl. The insets show the results of SDS-polyacrylamide electrophoresis of the separated peaks.

weights from 50,000 to 90,000 daltons were also buffer soluble. A small amount of ARP was detected in the soluble fraction by both SDS-polyacrylamide electrophoresis and immunoelectrophoresis. When the buffer-soluble apoprotein was applied to a heparin affinity column and eluted with a linear salt gradient, two fractions were eluted from the column (Fig. 1). The initial fraction was eluted at NaCl concentrations below 0.12 M and was found to contain no ARP in over one hundred chromatograms that had been run. A second protein fraction which eluted at NaCl concentrations from 0.35 to 0.4 M was found to be pure ARP by SDS-polyacrylamide electrophoresis (Fig. 1), immunoelectrophoresis, and NH$_2$-terminal analysis. The total amount of buffer-soluble ARP recovered and assayed by amino acid analysis was usually <1.5% of the mass of the delipidated apoproteins.

The buffer-soluble ARP recovered from the affinity column had a similar amino acid content (Table I) to the ARP previously described (21–24) and was immunochemically identical with the ARP purified by guanidine-Sepharose chromatography (2). The pure affinity-isolated ARP was run on a Multiphor Isoelectric Focus Unit (LKB Instrument, Inc.) in the pH 3–10 and 4–6 range, and it demonstrated at least five components with pI ranging from 5.8 to 6.3. NH$_2$-terminal analysis of the affinity-isolated ARP by the dansyl procedure with guanidine-Sephadex chromatography before hydrolysis (2) indicated lysine as the NH$_2$-terminal residue as was previously noted for the guanidine-prepared apoprotein (2).

An appreciable amount of the aqueous buffer-insoluble residue went into solution when it was reextracted with a similar buffer containing 5 M urea. When the urea-soluble fraction was applied to the heparin affinity column using the same NaCl gradient as previously, two major peaks were again observed. The second peak which eluted at NaCl concentrations from 0.17 to 0.20 M was found to contain only ARP (Fig. 2). A better separation of the peaks could be produced if a more shallow NaCl gradient (i.e. 0.05–0.5 M) were used (Fig. 4). The urea-soluble ARP recovered from the heparin affinity column usually accounted for 6.5–8.5%
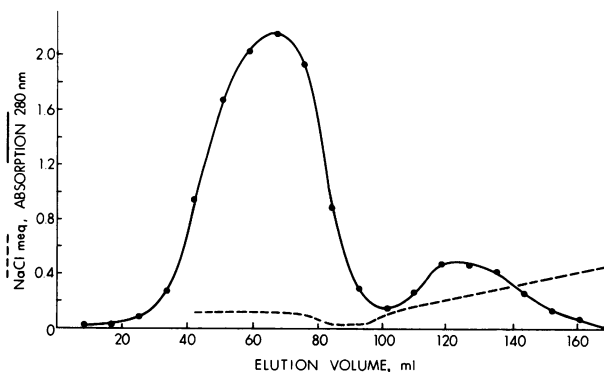

FIGURE 2 Heparin affinity chromatography of VLDL apoproteins soluble in 5 M urea, 0.12 M NaCl, and 2 mM phosphate. The gradient was the same as in Fig. 1.

of the delipidated apolipoproteins when assayed by amino acid analysis. The current estimates of total VLDL-ARP from VLDL prepared ultracentrifugally may be spuriously low because of ultracentrifugal losses. At present, it is impossible to relate the recovery of the ARP obtained from the affinity chromatography of these studies to the total ARP on VLDL prepared by gel filtration since this information is currently unavailable. The urea-soluble affinity-isolated apoprotein had a similar amino acid composition (Table I) and the same NH_2-terminal (lysine), molecular size, and immunochemical behavior as the buffer-soluble ARP. The residue after the urea buffer extraction was predominantly the apo beta protein but usually had traces of the ARP.

When native VLDL from fasting normal and hyperlipoproteinemic (type IV phenocopy) patients was applied to the affinity column, two distinct fractions of VLDL were eluted (Fig. 3). A small amount of lipoprotein equivalent to no more than one-fourth of total lipoprotein came off the column at NaCl concentrations below 0.12 M with the bulk of the lipoprotein eluted between 0.2 and 0.25 M. The unbound VLDL fraction contained the apo beta protein, the C apolipoproteins and variable quantities of larger (50–90,000 daltons) uncharacterized apoproteins by SDS-polyacrylamide electrophoresis and immunoelectrophoresis but only a trace of ARP (Fig. 3). The bound fraction had the apo beta and C apolipoproteins and the ARP was a prominent component. Both the bound and unbound lipoproteins rechromatographed in the same elution volumes where they were isolated. These two lipoprotein fractions also differed in their lipid contents (Table II), the bound fraction containing relatively more sterol ester and cholesterol and less triglyceride than the unbound.

TABLE II
Relative Lipid Composition of Heparin Bound and Unbound Whole VLDL Fractions

	Phospholipid	Free cholesterol	Cholesterol ester	Triglyceride
	% of total lipid*			
Normals				
Unbound	19.2 (1.8)	4.6 (.3)	4.2 (.1)	72.8 (1.7)
Bound	21.4 (.9)	7.6 (.2)	9.8 (.4)	61.8 (2.1)
Hyperlipoproteinemia (Type IV)				
Unbound	21.1 (2.1)	5.2 (.2)	4.9 (.1)	70.4 (1.9)
Bound	19.6 (1.6)	7.5 (1.)	12.2 (.3)	61.2 (1.1)

* These values represent the mean values of five individuals in each category. The bound and unbound lipoprotein cholesterol, cholesterol ester, and triglyceride values are significantly different at $P < 0.01$, by Student's t test.

Urea-soluble ARP isolated from the heparin affinity column was reapplied to the same column immediately after isolation (Fig. 4). The rechromatographed apoprotein was eluted in about the same elution volumes it was originally isolated. An aliquot of this apoprotein was derivatized with phenylglyoxal which produced an alteration of $\cong 75\%$ of the arginine residues (Table I) and reapplied to the same column. The derivatized protein was totally eluted in the unbound fraction (Fig. 4) with an extinction coefficient of the derivative greater than the parent protein. When native VLDL was similarly derivatized and reapplied to the column, all the lipoprotein eluted in the unbound fraction (Fig. 5) with no trace of bound lipoprotein as was previously noted for the native VLDL (Fig. 3).
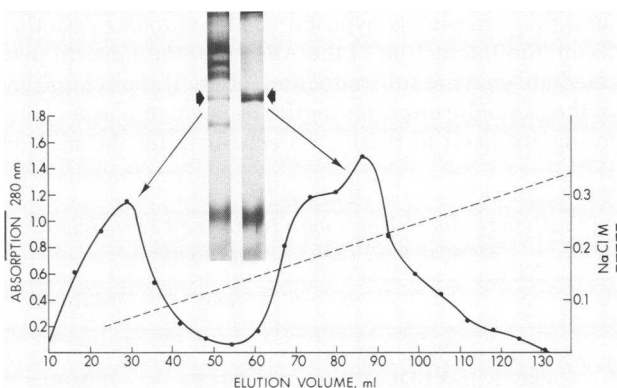

FIGURE 3 Heparin affinity chromatography of whole VLDL (9.5 mg protein) applied in 25 ml using the same conditions as in Fig. 1. The insets also show results of SDS-polyacrylamide electrophoresis of separated peaks after delipidation. The arrows at the side of each inset give the migration of standard ARP.
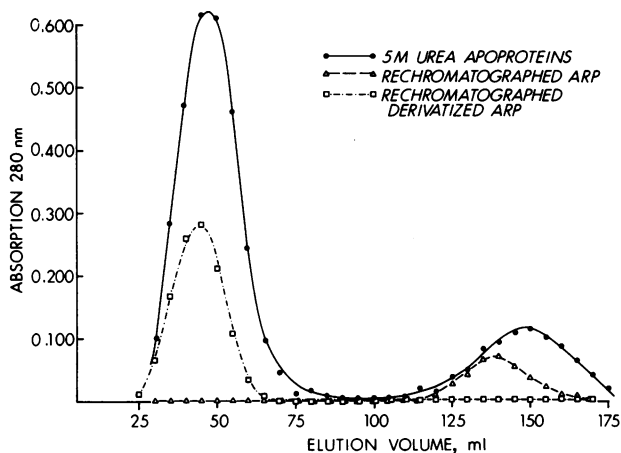

FIGURE 4 The VLDL apoproteins soluble in 5 M urea, 0.12 M NaCl, and 2 mM phosphate chromatographed on a heparin affinity column (●) one-half of the second peak (i.e. ARP) rechromatographed on the same column (△). The other half of the affinity isolated ARP derivatized with phenylglyoxal and reapplied to the same column (□).
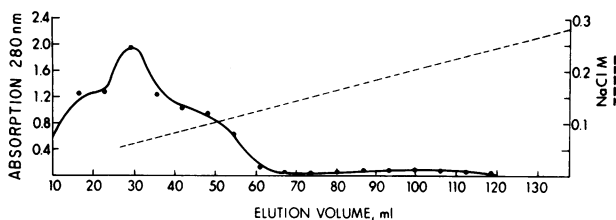
FIGURE 5 Heparin affinity chromatography of phenylglyoxal-derivatized whole VLDL (7.0 mg protein). The conditions are the same as in Fig. 2.

Dialyzed freshly prepared native VLDL (2 mg protein/ml) was incubated in 0.03 Tris, pH 7.5, 0.12 M NaCl buffer containing 0.03 μCi/ml of [35S]heparin (sp act 0.2 μCi/mg). The incubations were performed at 37°C for 3 h in a Dubnoff metabolic shaker after which the VLDL was isolated by ultracentrifugation and delipidated as described. The aqueous-soluble apoproteins were concentrated to 8.6 mg protein and 0.14 μCi [35S]heparin/ml by ultrafiltration on a UM-2 filter and evaluted by immunodiffusion using antisera to the apo-Ser, apo-Ala, apo-Glu, ARP prepared in rabbits by sequential immunization and commercially obtained antisera to high density lipoprotein. Although most of the protein in the aqueous-soluble apoproteins was the apo-Ala, apo-Glu, and apo-Ser apoproteins, the largest recovered heparin activities were in the precipitin lines obtained with the antisera to the ARP (Table III).

Native VLDL and phenylglyoxal derivatized VLDL at protein concentrations from 1 to 5 mg/ml were simi-

TABLE III
[35S]Heparin Activity in Immunoprecipitin Bands
of Aqueous-Soluble Apo-VLDL*

Antisera		
	cpm	%
Anti-apo-Ser	189	10.1
Anti-apo-Ala	200	10.7
Anti-apo-Glu	215	11.5
Anti-apo-HDL	175	9.3
Anti-apo-Arg (1)	517	27.7
Anti-apo-Arg (2)	841	45.1
Anti-apo-Arg (3)	743	40.2
Anti-heparin blank	166	8.9

* Immunodiffusion technique described in text using antisera to VLDL "C" apoproteins named according to the carboxy terminus. The anti Apo-Arg 1, 2, and 3 are three different lots of antisera to the ARP. The protein concentration of this aqueous-soluble apoprotein was 8.6 mg/ml with 0.14 μCi/ml of [35S]heparin. 1 μl of solution was applied to each of six antigen wells. Densitometry on the Coomassie Blue-stained apoproteins indicated the following apoprotein percentage: apo-Ser 14%, combined apo-Ala and apo-Glu 68%, apo-Arg 1.5%.

larly incubated with 0.03 μCi [35S]heparin/ml at 37°C for 3 h in a Dubnoff metabolic shaker using a buffer of 0.1 M N-ethylmorpholine at pH 5.5 to insure stability of the derivative (13). The lipoproteins were subsequently isolated by ultracentrifugation and the activities in the VLDL and the infranatant were assayed. The fresh native VLDL bound a mean of 89% of the radioactivity at these concentrations, whereas the phenylglyoxal-derivatized lipoprotein bound 30% of the heparin activity (Table IV). No differences in triglyceride recovery were found after centrifugation for the derivatized and native VLDL.

DISCUSSION

The triglyceride-rich lipoproteins of humans have a higher content of the ARP than any of the other plasma lipoproteins (2). This is not true for the rat which has much of its ARP in high density lipoproteins (25). Although the human triglyceride-rich lipoproteins carry most of this apoprotein, the functional role of the apoprotein in the metabolism of plasma triglyceride is completely unknown. The observation that the apoprotein is in particularly high concentrations in the triglyceride-rich lipoproteins which have appreciable contents of cholesterol ester (3, 23) has led to the postulate that this apoprotein has a role in the plasma transport of cholesterol. The increment of the apoprotein in the triglyceride-rich lipoproteins of humans after lecithin-cholesterol acyltransferase-deficient plasma has been exposed to an active lecithin-cholesterol acyltransferase system (4, 5) further supports a role for this apoprotein in plasma sterol metabolism.

The observation that the ARP binds heparin suggests a function for the apoprotein in the catabolism of plasma triglyceride. If heparin or another sulfated glycosaminoglycan is an integral part of the tissue lipoprotein lipase complex, as has been suggested (26), and this complex is exposed on the vascular endothelium, the interaction of the ARP with the heparin may mediate enzyme substrate interaction. It is obvious that if the catabolic enzymes for the removal of plasma glyceride are fixed in tissue, a mechanism for the fixation

TABLE IV
[35S]Heparin Incubations with Native and Phenylglyoxal-Derivatized VLDL (5 mg protein/ml)*

	Native	Derivatized
Recovered [35S]heparin activity in VLDL, %	89 (5)	30 (3)
Recovered [35S]heparin activity in infranatant, %	10 (1)	70 (3)

* Values represent the mean (standard deviation) of five incubations under conditions described in the text. The means for derivatized VLDL are significantly different at $P < 0.01$.

of plasma substrate is essential. Although it appears that lipoprotein lipase and heparin are intimately associated in tissue (26, 27), the circulating form of the enzyme (28) may have little affinity for heparin. Even if the tissue enzyme had no heparin affinity, the presence of a sulfated glycosaminoglycan in peripheral tissues would still act to anchor the substrate for subsequent catabolism.

The finding that a small amount of human triglyceride-rich lipoprotein in fasting plasma has almost no ARP on it suggests an analogy with the observations in our laboratory,[3] that chylomicrons of the rat lymph contained little ARP until they were exposed to the plasma. The same situation may be also true for the chylomicrons of humans, although the lipid composition of the unbound fraction is certainly not that of a postabsorptive chylomicron. However, it is recognized that triglyceride-rich lipoproteins are delivered through the lymph even in the fasting situation, and these have more of the characteristics of VLDL (29). The small unbound fraction may thus represent triglyceride-rich lipoproteins newly exposed to plasma that have not yet incorporated the ARP. We have observed that when this unbound fraction is reincubated in plasma, it now binds to the heparin affinity column and contains the ARP in a fashion analogous to rat lymph chylomicrons. Although evidence would indicate that the bulk of the VLDL in hepatic Golgi already contain the ARP (30), there may in fact be an appreciable fraction of the nascent VLDL that does not have the ARP. The heparin affinity column should prove to be an effective tool in isolating the VLDL fraction which contains the apoprotein from that which does not.

Recent in vitro incubations of fibroblasts with a swine lipoprotein containing the ARP also suggest another receptor function for the protein (31). This lipoprotein apparently has the same effect as the beta lipoprotein on cholesterol fibroblast metabolism (32) but contains no beta protein. It was suggested (31) that the ARP possibly serves the same function as the beta protein in this swine lipoprotein. The recognition that the beta protein fibroblast interaction can be disrupted with heparin (33) and that the ARP may be able to substitute for this function suggests that the receptor for cholesterol entry into the cell may be through sulfated glycosaminoglycan protein interaction.

The data presented only substantiate the fact that among the relatively soluble human VLDL apoproteins, it is solely the ARP which appreciably binds to heparin. In view of the similar binding of the human LDL and VLDL to heparin (34), it is quite probable that the apo beta protein also binds to heparin. The

insolubility of the apo beta protein in virtually all buffer systems makes the definition of this binding relationship difficult to attain. It is interesting that a small amount of human triglyceride-rich lipoprotein that contains the apo beta protein, but virtually no ARP (Fig. 3), does not bind to the affinity column. This might suggest that for the triglyceride-rich species the ARP is functionally more important in this interaction than is the beta protein.

The fact that heparin ARP binding is disrupted in high salt indicates an ionic interaction. The decrease of the binding in urea suggests that protein conformation importantly determines local charge densities which in turn determine the binding affinity. Iverius (8) has previously demonstrated a lack of VLDL or LDL heparin binding when the lipoprotein were acetylated suggesting an importance for the lysine residues. Our data would indicate that the arginine residues of ARP are also important for this interaction. The decreased heparin binding with arginine derivatizations may occur because of an alteration in protein charge, conformation, or both. The ability to derivatize the VLDL arginine residues by a technique which has little effect on primary amino groups provides more direct evidence that the heparin VLDL interaction is a protein-mediated one.

The ARP isolated by the affinity technique, either in aqueous buffers or in urea, differs somewhat from the chemical characteristics of the protein isolated on a guanidine-Sepharose system (2). The latter protein which had been carboxymethylated (2) was virtually completely insoluble in either an aqueous or urea buffer, whereas the affinity isolated proteins are at least readily soluble in urea. The molecular size, immunochemical reactivity, and NH_2-terminal residue was similar for the two preparations, but isoelectric focus of the affinity isolated material indicated a polymorphism in the pH range of 5.8–6.2, somewhat similar to that described by Utermann (24), whereas the guanidine preparation focused as a single basic band (2). Current preparations of guanidine-prepared ARP, when thoroughly dialyzed to rid them of guanidine, yield no urea-soluble material for isoelectric focus studies. It is possible that the previous basic focus of the guanidine-prepared apoprotein may have been a function of residual guanidine remaining with the protein, or possibly the different isoelectric focus techniques used (2).

ACKNOWLEDGMENTS

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