Changes in Apolipoproteins and Properties of Rabbit Very Low Density Lipoproteins on Induction of Cholesteremia†

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ABSTRACT: The cholesteremia induced in rabbits by a diet of the usual rabbit food plus 1% cholesterol is manifest in very high levels of serum very low density lipoproteins (VLDL, $d < 1.006$ g/cm^3). The low density lipoproteins are moderately elevated and the high density lipoproteins are decreased. The VLDL undergo changes in their content of the various apolipoproteins and lipids and in their physical properties. The electrophoretic mobility of the VLDL in agarose changes from pre-β to β; and the average particle size is larger. The normal VLDL contain on the average 8% protein, 17% phospholipids, and large amounts of triglycerides. The cholesteremic VLDL contain 4% protein, 13% phospholipids, very little glycerides, and large amounts of cholesteryl esters. Associated with these changes in VLDL properties and composition are changes in the relative amounts of several different apolipoproteins. One particular apolipoprotein, an arginine-rich glycoprotein that corresponds to one of the human apolipoproteins, is greatly increased so that it comprises about half of the protein moiety. The cholesteremic VLDL also contain significant amounts of a protein that appears to correspond to the human apoVLDL-Ser; and it contains also a fraction, ~40% of the total protein, that may correspond in part at least to the β proteins of human very low density lipoproteins. The cholesteremic VLDL contained very little if any of certain other apolipoproteins that occur in the normal triglyceride-rich lipoproteins. The rabbit arginine-rich apolipoprotein(s) occurs in multiple forms that differ in mobility in polyacrylamide gel electrophoresis. They were isolated by DEAE-cellulose chromatography. They contain glucosamine and galactosamine and are very similar to the human counterpart in amino acid composition and in conformation. The proteins are predominantly α helix in lipid-free form. Also isolated was the rabbit protein that corresponds to human apoVLDL-Ser in behavior in DEAE chromatography, in disc electrophoresis, and in its content of certain amino acids and its lack of tyrosine.

The very low density lipoproteins (VLDL)[1] of human plasma are mixtures varying in relative amounts of a number of different apolipoproteins. Normolipemic as well as hyperlipemic persons exhibit considerable individual differences in the proportions of these VLDL proteins and molecular species (Shore and Shore, 1973). It seems likely that the VLDL are of a dynamic composition that is determined, together with their concentration in plasma, by dietary, hormonal, genetic, and other regulatory factors superimposed upon the enzyme systems involved in the metabolism of the lipoproteins. It seems likely also that characteristic differences in the lipid compositions of the various VLDL species are associated with differences in the content of specific apolipoproteins. These apolipoproteins, which differ markedly in composition and conformation (Fredrickson *et al.*, 1971; Shore and Shore, 1972), undoubtedly differ also in their interactions with the phospholipids, triglycerides, cholesterol, and cholesteryl esters (or with mixed complexes of these lipids) in the VLDL.

From this study in rabbits, evidence is derived for a protein preferentially associated with cholesterol and/or cholesteryl esters of VLDL and for changes in VLDL composition induced by excess dietary cholesterol. A specific enrichment in an arginine-rich protein (apparently comparable to one of the human VLDL proteins) is associated with a marked change in lipid composition from VLDL rich in triglycerides to VLDL rich in cholesteryl ester. Changes in physical properties also accompanied these changes in composition.

Materials and Methods

Samples. Animals and Sera. Female New Zealand white rabbits (initially 6–7 lb each) were divided into two groups, one of which was fed Purina[2] rabbit pellets and the other, the pellets plus 1% cholesterol. A solution of cholesterol in peroxide-free ether was slowly added to the pellets and the solvent was allowed to evaporate. Seven rabbits were fed cholesterol; two were bled after 2 days and five were bled at 10 days and again at 24 days; there was one control rabbit for each cholesterol-fed rabbit. About 10 ml of blood per animal (unfasted) was taken from the ear vein at each interval. A small volume of blood for agarose electrophoresis was taken from all the animals at the beginning of the experiment. Each of the serum and lipoprotein samples was analyzed individually except as noted, *e.g.*, for isolation of proteins by ion exchange chromatography.

Serum Lipoproteins. Isolation of the lipoproteins was begun on the same day as the bleeding. The VLDL ($d < 1.006$ g/cm^3), LDL_2 (d = 1.006–1.019 g/cm^3), LDL_1 (d = 1.019–1.065 g/cm^3), and the HDL (d = 1.081–1.21 g/cm^3) fractions were isolated sequentially by centrifugation in solutions adjusted with NaCl to the appropriate densities (Shore, 1957). EDTA at 0.0008 M and 0.01 M Tris buffer at pH 7.5 were present throughout. We frequently have examined the lipoproteins with respect to the disc electrophoresis patterns and amino acid composition of their apolipoproteins after one isolation procedure and then again after recentrifugation of the lipoproteins. We found that the VLDL could be isolated ade-

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[1] Abbreviations used are: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

[2] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Atomic Energy Commission or the University of California to the exclusion of others that may be suitable.

quately from diluted (4–8-fold) serum by one centrifugation; this is desirable because some apolipoproteins can be lost in part (Herbert *et al.*, 1973) and lipoprotein denaturation could occur on repeated centrifugation.

The concentrations of the various lipoprotein fractions in serum were estimated from the quantity of lipoprotein (total lipids plus apolipoproteins) isolated from a known volume of serum.

Analytical Procedures. Agarose Electrophoresis. Aliquots of whole serum, isolated lipoproteins, and infranatant after removal of the VLDL were subjected to agarose electrophoresis (Hatch *et al.*, 1973) on commercially available plates at pH 8.6. The lipoproteins were stained with fat red 7B.

Lipid Analysis. The total serum cholesterol levels were determined by the methods of Crawford (1958) and Mann (1961). Total lipids of the isolated lipoproteins were determined gravimetrically. Phosphorus analyses were carried out by the method of Chen *et al.* (1956) on the extracted lipids and VLDL samples. The two kinds of samples gave the same phospholipid content (assumed to be 25 times the phosphorus content) after correction for per cent protein. Similarly, the distribution of triglycerides, cholesterol, and cholesteryl esters on thin-layer chromatograms was the same irrespective of whether the extracted lipids or intact lipoprotein was taken for analysis. The lipids of the isolated VLDL were separated as described by Nelson (1967) by one-dimensional thin-layer chromatography on a glass plate coated with silica gel. The solvent was petroleum ether–diethyl ether–acetic acid, 85:15:2. This solvent was also used for a preliminary washing of the plate, which was then air-dried, activated for 30 min at 120°, and finally cooled in air before the samples were applied. After chromatographic separation of the lipids, the plates were air-dried, sprayed with 18 N H_2SO_4, and then heated on a hot plate to char the lipids.

Delipidation of Lipoproteins. The lipids were extracted from the lipoproteins at a concentration of ~10 mg/ml in aqueous solution at 2–4°. The undialyzed lipoproteins at pH 7.6 were extracted several times with diethyl ether, then dialyzed against cold degassed water, and extracted further at pH 4 and at 2–4° with ether–ethanol mixtures (with increasing ethanol concentration from 10 to 30% by volume). Precautions were taken to recover small amounts of protein carried over in the lipid extracts. The delipidated, dialyzed protein was water soluble; amino acid analyses indicated it was the same in composition and quantity as the protein moiety of the undialyzed, ether-extracted material.

Disc Electrophoresis. The delipidated protein moieties so obtained were subjected to disc gel electrophoresis, essentially as described by Davis (1964), in polyacrylamide gels (10%) containing 8 M urea. The samples were preincubated in 8 M urea at room temperature for 0.5–1 hr; all of the protein was soluble. Tris-glycine buffer at pH 8.3 and a current of 2.5 mA/tube were used.

Fractionation of Apolipoproteins. The entire protein moiety (*i.e.*, no losses as insoluble protein, etc.) of the VLDL was taken for ion exchange chromatography on DEAE-cellulose. The procedure was that used for fractionation of human VLDL proteins (Shore and Shore, 1970, 1973). For isolation of the proteins not eluted from DEAE-cellulose, the lipid-free protein in 7 M urea containing 0.01 M Tris-HCl at pH 8.0 was gel filtered on Sephadex G-150 (0.9 × 75 cm) equilibrated with the same solvent; this protein fraction was eluted first.

Amino Acid and Amino Sugar Analyses. Amino acid analysis was by the 4-hr methodology described in Beckman Instruments' (Palo Alto, Calif.) technical bulletin A-TB-033. Measured aliquots of the protein solutions were lyophilized; the protein was then hydrolyzed in constant boiling HCl containing mercaptoethanol (1 μl/2 ml) at 110° for 22 hr after sealing the evacuated and degassed sample. Half-cystine was determined as cysteic acid as described by Moore (1962); the protein was oxidized with performic acid and hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 110° for 18 hr. Tryptophan was estimated spectrophotometrically by the method of Bencze and Schmid (1957) and also by the method of Liu and Chang (1971) except that the 3 N *p*-toluenesulfonic acid was replaced by 4 N methanesulfonic acid. The latter method also permitted estimation of glucosamine, galactosamine, and mannosamine. Correction factors of 7, 5, and 10% were applied to cysteic acid, threonine, and serine, respectively.

Since measured aliquots of protein solutions were taken for amino acid analysis, the data (in μmol/known volume) together with the formula weights of the amino acid residues could be used for estimation of protein concentration. The per cent protein in the lipoproteins was estimated from the amount of protein in the sample (protein concentration times total volume of sample) and the weight of lipids extracted from the lipoprotein sample.

Electron Microscopy. Freshly isolated VLDL samples were taken for electron microscopy; 1 ml of isolated lipoproteins (15–25 mg/ml) was dialyzed 3 hr on a rapid dialyzer against 600 ml of 0.95% ammonium acetate–ammonium bicarbonate (2:1) solution containing 0.0005% EDTA and adjusted to pH 7.3 with CO_2. Immediately after dialysis, the lipoproteins were diluted to about 0.8 mg/ml and an aliquot was mixed with an equal volume of 2% potassium phosphotungstate at pH 7.4. In one experiment, the undialyzed cholesteremic VLDL was diluted 30-fold with the buffer; the result was essentially the same as for the dialyzed sample. Without delay, a gross drop of the mixture was placed on a carbon-parlodion-coated grid, where it was allowed to remain for 20–60 sec. The drop was then removed by touching with filter paper, and the grid was examined in the electron microscope at 80 kV potential. Micrographs were made at a magnification of 40,000 and subsequently enlarged.

Results

Cholesterol-enriched diets promptly induce cholesteremia in rabbits (Schumaker, 1956; Kritchevsky *et al.*, 1969). In two of our rabbits that were bled after 2 days on the diet, the cholesteremia was already evident, with VLDL levels of ~3 mg/ml of serum. In five other rabbits, the serum VLDL rose from the normal very low levels (<1 mg/ml) to averages of 18 and 29 mg/ml after 10 and 24 days, respectively, on the cholesterol-enriched diet. The LDL_1 and LDL_2 at 2–3 and 5–7 mg/ml of serum, respectively, were also elevated but to a lesser extent, *ca.* two- to fourfold for LDL_1 and five- to eightfold for LDL_2. The serum HDL were considerably reduced. Total serum cholesterol (free and esterified) values ranged from 60 to 80 mg/100 ml in the normal animals and 1300 to 2000 mg/100 ml and 1700 to 3500 mg/100 ml, respectively, in 10-day and 24-day cholesteremic rabbits.

The cholesteremic VLDL differed from the VLDL of the control rabbits in electrophoretic mobility, lipid composition, relative amounts of the various apolipoproteins, and appearance in electron microscopy after negative staining of the lipoproteins with potassium phosphotungstate.

Agarose Electrophoresis. The VLDL after cholesterol feeding were almost entirely of β mobility in agarose electro-

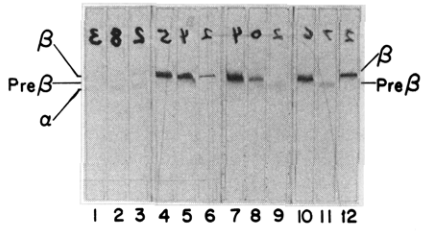

FIGURE 1: Agarose electrophoresis patterns of rabbit serum lipoproteins stained with fat red 7B. Patterns 1, 2, and 3 are normal sera; the α or HDL band is the predominant band and there is a narrow band in the pre-β position and a darker band in the β position in all of the chow-fed rabbit sera. Patterns 4, 5, and 6 are the serum, VLDL, $d > 1.006$ fractions, respectively, after 10 days of the cholesterol-enriched diet; 7 and 8 are serum and VLDL (diluted), respectively, after 24 days of the cholesterol diet; the α bands in cholesteremic sera are very faint. Patterns 9 and 11 are normal VLDL (concentrated); 10 and 12 are cholesteremic VLDL and LDL_2 (concentrated), respectively.

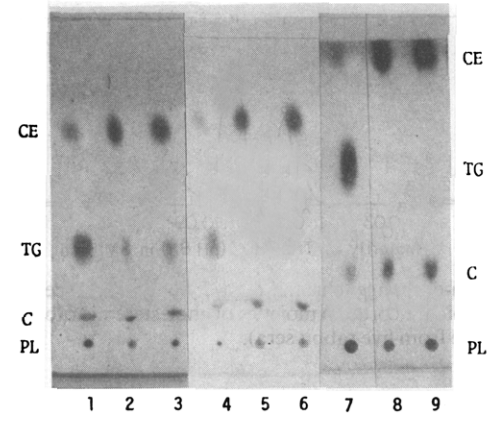

FIGURE 2: Thin-layer chromatography of the lipids of VLDL isolated from normal and cholesteremic rabbit sera: 1, 4, and 7, normal VLDL; 2 and 3, 2-day cholesteremic VLDL; 5 and 6, 10-day cholesteremic VLDL; 8 and 9, 24-day cholesteremic VLDL; CE, cholesteryl esters; TG, triglycerides; C, cholesterol; PL, phospholipids.

phoresis, whereas in the controls they were mainly of pre-β mobility (Figure 1). This change in VLDL was seen in each of the five rabbits both at 10 and 24 days on the cholesterol-enriched diet. The agarose electrophoresis patterns of the serum lipoproteins of normal rabbits (*i.e.*, prediet and control rabbits, with normal lipid levels) resemble those of normal humans (Hatch *et al.*, 1973) except that the serum levels, particularly of the β-lipoproteins or LDL, are somewhat lower (Figure 1, patterns 1–3); there are low levels of VLDL, almost entirely of pre-β mobility (patterns 9 and 11). In cholesteremic rabbits (Figure 1, patterns 4–8, 10, and 12) the β component is greatly increased, mainly due to increased VLDL, and the HDL or α-lipoproteins are low.

Composition of Rabbit VLDL. In the cholesteremic rabbit, the VLDL were changed from triglyceride-rich to cholesterol-rich (particularly in cholesteryl esters) particles as shown by thin-layer chromatography of the lipids (Figure 2). These compositional differences were seen in each of the five cholesteremic rabbits analyzed separately. At the 10- and 24-day intervals the triglyceride content of the VLDL was very low (Figure 2, patterns 5, 6, 8, 9); even after 2 days of the cholesterol diet, the cholesteryl ester: triglyceride ratio was much higher than before, but significant amounts of triglyceride were present (patterns 2 and 3). The triglyceride-rich VLDL contained an average of 7.5% protein (range 6.9–8.3, excluding the carbohydrate moiety) and 17.2% phospholipid (range 16.4–18.4); the cholesteryl ester rich VLDL contained an average of 3.9% protein (range 3.7–4.1) and 13.2% phospholipid (range 12.5–13.8). The phospholipid-to-protein ratio by weight, 3.4, was constant among the different samples of cholesteremic VLDL.

Polyacrylamide Gel Electrophoresis of Apolipoproteins. Associated with these changes in electrophoretic mobility and lipid composition of the VLDL was a marked change in protein composition. One particular apolipoprotein was greatly increased in proportion to the rest of several proteins that are normally present in VLDL. This protein(s), which occurs in mulitple forms, corresponds in Figure 3 to bands R2 and R3 of the disc electrophoresis patterns of the delipidated VLDL in polyacrylamide gels containing 8 M urea. The patterns shown in Figure 3 are of: (1) unfractionated VLDL and LDL of normal and cholesteremic rabbits, (2) apolipoproteins isolated by ion exchange chromatography of delipidated VLDL of cholesteremic rabbit serum, and, for comparison, (3) a pattern of the delipidated VLDL of human serum. The patterns of delipidated VLDL are representative of all the experimental animals whose VLDL were analyzed separately. Comparable amounts of unfractionated VLDL protein (~100 μg) were taken in each case; yet the cholesteremic samples (*e.g.*, pattern 2 in Figure 3) always had relatively more of the R2 and R3 proteins than the VLDL of control animals (*e.g.*, pattern 1). The cholesteremic rabbit apoLDL_2 (pattern 3) contains proportionately less of the R2 and R3 proteins than the cholesteremic VLDL; LDL_1 (patterns 4 and 5) contains relatively little. All of the isolated rabbit proteins shown in patterns 6–11 of Figure 3 were derived from the pooled apolipoproteins of five cholesteremic animals. For the LDL patterns, ~200 μg of protein was taken. No rabbit serum albumin was seen in any of the disc electrophoresis patterns; it migrates slightly faster than the protein of band R3 in Figure 3, but it is well separated from the apolipoproteins and is easily seen at a level of 1% of the total protein (*i.e.*, 1 μg of albumin in the apolipoprotein sample). Nor do the major proteins of rabbit HDL correspond to any of the apolipoprotein bands in the patterns shown in Figure 3. Subsequent work described below suggests that bands R2 and R3 in rabbit patterns 1–5 and 7–11 correspond to band H2 in the human pattern 12 and that rabbit band R1 may correspond to human band H1 in Figure 3. The latter is a protein (apoVLDL-Ser) with amino-terminal threonine and carboxyl-terminal serine. Band H2 is a protein that is rich in arginine and glutamic acid and is the most helical in conformation of the known apolipoproteins in lipid-free form (Shore and Shore, 1970, 1972).

Fractionation of VLDL Proteins. Apolipoprotein components corresponding to each of the polyacrylamide gel bands

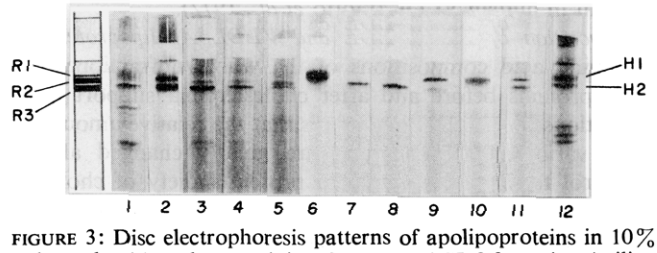

FIGURE 3: Disc electrophoresis patterns of apolipoproteins in 10% polyacrylamide gels containing 8 M urea (pH 8.3; stain, Aniline Blue Black): pattern 1, normal rabbit VLDL; 2, 3, and 4, cholesteremic VLDL, LDL_2, and LDL_1; 6, isolated R1 (DEAE fraction 1); 7, 8, 9, and 10, isolated components of R2 and R3 (DEAE fractions 4, 5, 6, and 7, respectively); 11 = 8 + 9; 12 is human VLDL.

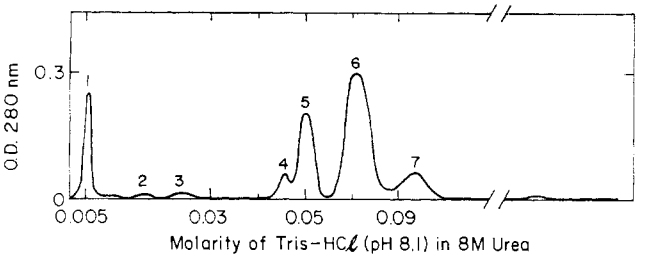

FIGURE 4: Ion-exchange chromatography on DEAE-cellulose of the whole delipidated protein moieties of cholesteremic rabbit VLDL (pooled sample from five rabbit sera).

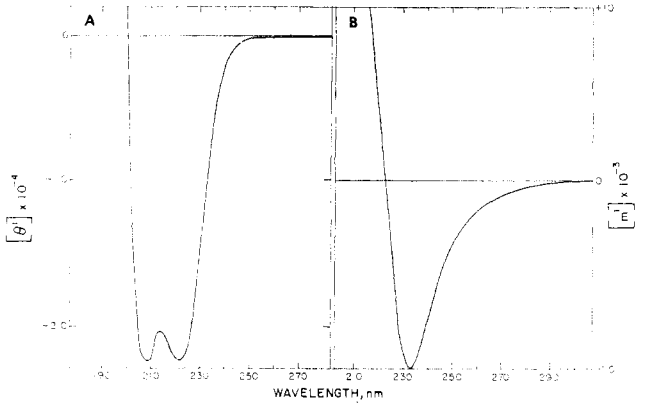

FIGURE 5: CD (A) and ORD (B) spectra at pH 7.4 of the arginine-rich glycoproteins isolated from rabbit VLDL.

(R1, R2, and R3 of Figure 3) in the pattern of unfractionated cholesteremic apoVLDL were isolated by ion exchange chromatography of the lipid-free protein moiety on DEAE-cellulose under conditions used for fractionation of the human VLDL proteins (Shore and Shore, 1970, 1973). Pooled 10-day and pooled 24-day cholesteremic VLDL proteins (6.6 and 14.3 mg of protein, respectively), chromatographed separately, gave essentially the same results. Aliquots of the isolated, dialyzed proteins corresponding to DEAE fractions 1, 4, 5, 6, and 7 of Figure 4 gave disc electrophoresis patterns 6, 7, 8, 9, and 10, respectively, in Figure 3; DEAE peak 1 corresponds to electrophoresis band R1 in the pattern of unfractionated cholesteremic VLDL (pattern 2 and the schematic drawing on the left in Figure 3); peaks 4 and 5, to band R3; and peaks 6 and 7, to band R2. Electrophoresis of a mixture of the proteins from DEAE fractions 5 and 6 gave two bands (pattern 11 of Figure 3) that simulate the R2 + R3 group in the pattern of the unfractionated apolipoprotein (pattern 3 and schematic drawing on left) from which they were derived. The rabbit apolipoprotein of DEAE peak 1 (electrophoresis band R1), like the human protein apoLP-Ser of band H1, was not adsorbed to the DEAE-cellulose column. It is a minor component of the cholesteremic VLDL protein, amounting to about 5% of the total protein applied to the column. The rabbit arginine-rich protein(s), like its human counterpart, was eluted in several different fractions (4, 5, 6, and 7 of Figure 4) but at somewhat higher ionic strength. These arginine-rich proteins are a major fraction (about 50%) of the total protein of cholesteremic VLDL. Other proteins, ~40% of the total, were not eluted from the DEAE-cellulose column. For analysis, this fraction (*i.e.*, the proteins that remain in the sample gel in disc electrophoresis) was separated by gel filtration from the proteins that are seen in the separating gel in disc electrophoresis, *i.e.*, those that were eluted from DEAE. This fraction (sample gel proteins) comprised 40–50% of the total proteins of the cholesteremic VLDL that was gel filtered; it, like the β moiety of human VLDL under comparable conditions (Shore and Shore, 1972), was not retarded in a column of Sephadex G-150. Possibly they correspond to the β-protein moiety that is present in human VLDL (Gotto *et al.*, 1972).

Composition of Rabbit LDL and VLDL Apolipoproteins. The amino acid compositions of the whole unfractionated VLDL proteins before and after cholesteremia support the observations by disc electrophoresis that the relative amounts of the various apolipoproteins are greatly changed after cholesterol feeding. Although the protein moiety of cholesteremic VLDL is heterogeneous, the amino acid compositions of all the samples at 10 and 24 days were very similar to that given in Table I. The compositions of the unfractionated protein of triglyceride-rich normal VLDL varied somewhat, particularly in glycine, alanine, and leucine content, but in no case was the arginine value >36 mol/10^3 mol. From the amino acid data of the VLDL (Table I), it is evident that the percentages of arginine and glutamic acid are considerably increased. This is due, in large part, to increased amounts of the proteins designated R2 and R3 (Table I), which are the major fractions from ion exchange chromatography of cholesteremic VLDL proteins. These arginine-rich proteins of the rabbit are very similar in amino acid composition to the human protein designated H2 in Table I and they contain glucosamine and galactosamine. After disc electrophoresis, they give a positive reaction with the periodate–Schiff test described by Zacharius *et al.* (1969). The rabbit apolipoprotein designated R1 in Table I (band R1 in Figure 3 and peak 1 in Figure 4) appears to correspond to human apoVLDL-Ser (H1 in Figure 3 and Table I). They are similar in that they contain no tyrosine and in their content of lysine, serine, glutamic acid, and methionine (and in that they are isolated by the same conditions and migrate similarly on electrophoresis in 10% polyacrylamide gels), but they differ considerably in content of some amino acids. Table I also gives the composition of the fraction of VLDL proteins that was separated by gel filtration (but not eluted from the DEAE-cellulose column). Also given in Table I are the compositions of rabbit LDL_1 and LDL_2.

Circular Dichroism and Optical Rotatory Dispersion (ORD) Spectra of the Arginine-Rich Apolipoprotein. The significance of the existence of multiple forms of an apolipoprotein for the structure and metabolism of the lipoproteins is not known. However, the differences (possibly in a carbohydrate moiety) do not appear to change the conformation in any way that can be detected in their circular dichroism spectra. Spectra of the different forms of the rabbit arginine-rich proteins are essentially the same as shown in Figure 5A and are very similar to the human counterparts (Shore and Shore, 1972). These spectra of these isolated, lipid-free proteins resemble those given by proteins containing considerable amounts of α helix; comparison with the spectrum of poly(α-L-glutamic acid) (Jirgensons, 1969) suggests that about two-thirds of the protein molecule is helical. The percentage helix is somewhat greater at pH 8.5 and somewhat less at pH 6.5 than at pH 7.4. The ORD spectrum of the arginine-rich protein, shown in Figure 5B, is consistent with a predominantly α-helical conformation (Jirgensons, 1969).

VLDL Particle Size. These cholesteremic VLDL that are greatly enriched in cholesteryl esters and the arginine-rich protein(s) are mainly very large particles. They are very turbid in solution but they do not float as do chylomicrons, possibly due in part to their greater buoyant density caused by a high cholesteryl ester content instead of a high triglyceride content; nor do they stay at the origin in agarose electrophoresis as

TABLE I: Amino Acid Composition (moles/10^3 moles of amino acids) of Rabbit Serum Lipoproteins and Apolipoproteins Isolated from VLDL.

	Normal		Cholesteremic			VLDL Subfractions[a]					
						Rabbit				Human	
	VLDL	LDL_1	VLDL	LDL_2	LDL_1	β(?)	R1	R2	R3	H1	H2
Lys	64	75	64	67	73	73	149	44	43	154	49
His	17	18	14	18	19	19	16	10	9	0	11
Arg	34	39	69	52	45	34	33	101	102	54	106
Asp	99	97	76	88	91	93	63	50	48	90	48
Thr	57	63	54	60	61	61	114	31	30	45	39
Ser	91	82	68	72	78	104	102	58	57	126	60
Glu	134	146	178	154	146	139	161	227	229	159	228
Pro	48	40	37	38	39	39	23	32	33	14	29
Gly	105	52	55	48	52	69	23	59	59	29	59
Ala	83	73	91	79	75	71	42	118	120	54	106
½-Cys	9		6	7			0	0	0	0	0
Val	49	52	56	56	55	50	16	62	62	35	67
Met	16	20	23	20	21	15	16	30	30	17	23
Ile	38	52	34	49	51	50	81	10	9	48	11
Leu	82	118	113	119	116	107	70	109	112	104	108
Tyr	26	27	23	27	27	29	0	15	15	0	14
Phe	38	44	33	41	41	41	81	15	15	48	14
Trp	+	+	+	+	+		+	26		24	28

[a] Rabbit apolipoproteins R1, R2, and R3 were isolated by ion exchange chromatography and correspond to the bands in polyacrylamide gel electrophoresis patterns of Figure 3; the β(?) fraction was separated by gel filtration and corresponds to protein(s) not entering the separating gel in disc electrophoresis. Human apolipoprotein H1 appears to correspond to R1; and H2, to R2 and R3.

chylomicrons do. Electron micrographs of the particles after negative staining with potassium phosphotungstate (Figure 6) indicate that the cholesterol-rich VLDL are on the average larger than the triglyceride-rich VLDL. The latter are quite variable in size, as has been noted for human VLDL (Lossow *et al.*, 1969).

Discussion

Other investigators have reported that the serum lipoproteins of cholesteremic rabbits are very rich in cholesteryl esters and low in triglycerides. This is so whether the rabbits are given cholesterol or cholesterol plus fat, *e.g.*, cottonseed oil or corn oil (Schumaker, 1956; Kritchevsky *et al.*, 1969). Schumaker (1956) reported also that the major serum lipoprotein species were S_f 5–15 and VLDL of $S_f > 40$ were far lower in concentration than the S_f 5–15 lipoproteins. Our experiments indicate that the major species are large VLDL particles rich in cholesteryl esters and very low in triglycerides. The VLDL of our control rabbits were triglyceride rich, as has been reported by Mills and Taylaur (1971), who investigated the serum lipoproteins of 18 animal species.

The liver appears to be the major source of the cholesteryl esters of the cholesteremic rabbit VLDL, whereas in man the esters are formed mainly in plasma (Rose, 1972). In spite of this difference, both human and rabbit VLDL contain remarkably similar forms of an arginine-rich apolipoprotein, the protein that is preferentially increased in the cholesteremic VLDL.

The relatively large amount of the arginine-rich apolipoprotein in the cholesteremic VLDL is unlikely to be due only to the absence of certain VLDL proteins that are present in triglyceride-rich VLDL; it appears to reflect also an increase in the absolute amount of arginine-rich protein per particle. The decrease in the percentage of total protein from 8 to 4% that is associated with the change from triglyceride-rich to cholesteryl-ester-rich VLDL may be related to the larger average particle size in the latter. Lossow *et al.* (1969) observed that increased surface to volume ratio as particle size decreases is approximately compensated by an increase in the weight percentage of the protein.

Our observations suggest that the arginine-rich apolipoprotein, which is specifically larger in relative amount in the cholesteremic VLDL, is one of several apolipoproteins primarily involved with cholesterol transport and metabolism. The parallel increase in cholesteryl esters and this arginine-rich protein(s) suggest that it may be a binding protein specific for cholesteryl esters (or a complex of these esters with phospholipids and/or cholesterol). Other VLDL proteins such as

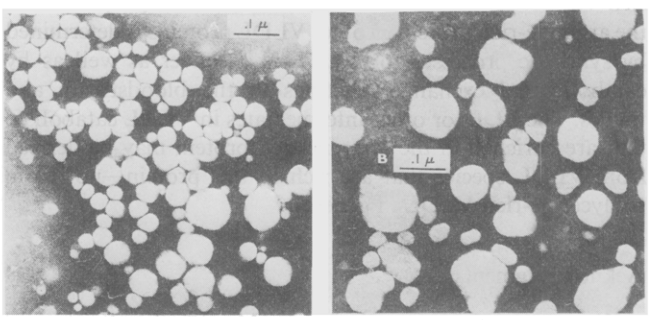

FIGURE 6: Electron micrographs of VLDL from normal rabbit serum (A) and cholesteremic rabbit serum (B).

the lipoprotein lipase activator protein (Havel *et al.*, 1970; LaRosa *et al.*, 1970), which in lipid-free form is predominantly random coil in conformation, or its phospholipid complex, may preferentially bind triglycerides. No role for the arginine-rich protein as a cofactor in enzyme systems of lipid metabolism has yet been identified. It does not affect lipolysis by lipoprotein lipase (Havel *et al.*, 1970) and the cholesteremic VLDL are not affected by the injection of heparin (Schumaker, 1956). It does not appear to affect the esterification of cholesterol by lecithin–cholesterol acyltransferase (Fielding *et al.*, 1972), but possibly it could be involved indirectly as an acceptor or transport protein for cholesteryl esters.

It is not yet clear why excess dietary cholesterol in rabbits leads primarily to an elevation in plasma VLDL and not in plasma LDL_1 (also cholesterol-rich lipoproteins). The rate-limiting step appears to be in the catabolism of these lipoproteins or lipid complexes containing the arginine-rich protein, rather than in the degradation of the LDL presumably derived from VLDL (Bilheimer *et al.*, 1972) and containing little of the arginine-rich protein. This diet-induced situation in the rabbit may be analogous to that in human type III hyperlipoproteinemia (primary dis-β-lipoproteinemia, *Xanthoma tuberosum*, floating β disease) (Gofman *et al.*, 1954; Fredrickson and Levy, 1972; Hazzard *et al.*, 1970; Havel and Kane, 1973). In type III disease, the plasma contains abnormally high levels of LDL_2 and VLDL, but not LDL_1, that are rich in cholesteryl esters and relatively poor in triglycerides (though not to the extent in the cholesteremic rabbit VLDL), are predominantly β in mobility in agarose or paper electrophoresis, and are relatively enriched in the arginine-rich apolipoprotein (Havel and Kane, 1973; Salel *et al.*, 1973).

It has been suggested that these lipoproteins that are elevated in type III plasma are "remnants" (*i.e.*, products) of the catabolism of triglyceride-rich particles (Havel and Kane, 1973). This may be so, but certain of our observations are not obviously reconcilable with this view. In type III and hypothyroid subjects (Shore *et al.*, 1973) and in cholesteremic rabbits LDL_2 (probably intermediates or "remnants" in the catabolism of VLDL) has relatively more of the arginine-rich protein than usual. But LDL_2 often contain very little of the arginine-rich protein and relatively more of other VLDL proteins, *e.g.*, apoVLDL-Ala or apoVLDL-Ser. If the arginine-rich protein is present in relatively small amounts in the serum VLDL, it is likely to be present only in very small amounts in the LDL_2, even if the LDL_2 are elevated (Salel *et al.*, 1973; Shore *et al.*, 1973; V. G. Shore and B. Shore, unpublished observations). The rabbits fed excess cholesterol appear to synthesize considerable quantities of triglyceride-poor VLDL that are very rich in this protein, even richer than the type III lipoproteins. These rabbit particles are as large or larger than the triglyceride-rich VLDL—not smaller as one would expect if they were intermediates in the synthesis and/or catabolism of triglyceride-rich VLDL. It seems more likely to us that the proportion of the arginine-rich protein to other VLDL proteins is determined by synthetic processes for VLDL in the liver as well as by catabolic and exchange reactions in the bloodstream and tissues. The LDL_2 or other intermediates in VLDL catabolism that are enriched in the arginine-rich protein may be derived from VLDL species that are rich in this protein—not from triglyceride-rich VLDL in general.

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Two Pictures of a Lipid Bilayer. A Comparison between Deuterium Label and Spin-Label Experiments†

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ABSTRACT: The segmental order parameters in a lipid bilayer are measured by means of deuterium magnetic resonance and by means of spin-labels. The deuterium results show an almost constant order parameter over most of the chain, which decreases rapidly near the last three carbon atoms. In contrast, the spin probes reveal a continuous decrease of the order parameter. The deuterium results show the physical state of the unperturbed bilayer. They can be interpreted in terms of a kink model for the bilayer structure. The spin-labels reveal the response of the bilayer on a small perturbation.

During the last 5 years lipid bilayers have been studied extensively by means of spin probes (*cf.* review by Jost *et al.* (1971)). Deuterium magnetic resonance (dmr) of selectively deuterated lipid samples provides similar structural information but with the advantage of not perturbing the liquid crystalline phase (Seelig and Niederberger, 1974). Our previous report dealt with some methodological aspects of deuterium labeling. We present here the results of a lipid bilayer study including both deuterium labels and spin-labels. Two different pictures are obtained for the structure of the lipid bilayer depending on the method used.

Experimental Section

The bilayer system was a liquid crystalline phase of the following chemical composition: sodium decanoate (32 wt %), decanol (38 wt %), and water (30 wt %), where the decanoic acid was replaced by its selectively deuterated analog. Nine bilayer phases of the above mentioned lipid composition were prepared, each containing a CD_2 group (CD_3 for the last C atom) at a different position in the hydrocarbon chain of the decanoic acid. In all samples, the decanol molecule was replaced by 1,1-dideuteriodecanol, so that the residual quadrupole splitting of this alcohol could be used as an internal standard. The deuterated substances were synthetized by Kolbe electrolysis (Nguyên-Dinh-Nguyên, 1968).

The bilayer phase (400 mg) was prepared in a sealed ampoule and placed in a 10-mm nuclear magnetic resonance (nmr) tube. The dmr measurements were performed at ambient temperature (~28°) with a Bruker FFT-HX 90-spectrometer operating at 13.8 MHz. Our previous instrumentation was improved by a Bruker BNC-73 computer with a spectral range of 50 kHz (compared to 25 kHz in the earlier experiments). A 90° pulse of 18 μsec width was used. In the liquid crystalline bilayer the deuterium nucleus has a rather short T_1 relaxation time, so that 9000 free induction decays could be accumulated in 30 min.

Results

Typical dmr spectra are shown in Figure 1. The molar ratio decanol–sodium decanoate in this bilayer is about 1.5, and therefore the more intense signal is due to 1,1-dideuteriodecanol. The smaller peak arises from deuterated decanoic acid. The residual quadrupole splitting $\Delta\nu_p$ (= separation of the corresponding doublets) is constant for 1,1-dideuteriodecanol, but changes for the decanoic acid depending on the position of the deuterium in the chain. The residual quadrupole splittings are listed in Table I. The experimental error amounts to approximately 0.5–3%. From the residual quadrupole splittings the order parameter of the deuterium bond S_{CD} and that of the chain segment S_{mol} can be calculated as described previously (Seelig and Niederberger, 1974). These results are also given in Table I. A quadrupole constant of 170 kHz, characteristic of deuterated paraffins (Burnett and Muller, 1971), was used for all CD bonds regardless of the position in the chain.

Following the dmr experiments the bilayer phases were doped with a small amount of stearic acid spin probes. The spin probes and the experimental procedures have been described previously (Seelig, 1970; Seelig *et al.*, 1972). The order parameter S_3 measures the average orientation of the chain segment at the position of the NO group. In principle, both order parameters S_{mol} and S_3 should have the same value for a given chain segment.[1] The spin-label results are listed in the last column of Table I.

Discussion

In Figure 2 we have plotted the order parameters S_{mol} and S_3 as a function of the chain position. There is a distinct

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[1] Deuterium magnetic resonance is sensitive to slower motions than electron spin resonance. This plays no role in our system but may be important for phospholipids as has been pointed out to us by Professor H. M. McConnell.