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NH_2-terminal amino acids of the serum lipoproteins of normal and hypercholesterolemic rabbits*

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SUMMARY

Aspartic acid is the major and glutamic acid and serine are minor NH_2-terminal amino acids of the lipoproteins of rabbit serum. No NH_2-terminal amino acids other than these are found after prolonged cholesterol feeding. The hyperlipoproteinemia accompanying the feeding is not characterized by any significant changes in the NH_2-terminal amino acid composition of the density (d) $<$ 1.007, 1.007 to 1.019, or 1.065 to 1.20 lipoproteins. However, in the d $=$ 1.019 to 1.065 lipoproteins, the percentages of NH_2-terminal glutamic acid and aspartic acid seem to increase and decrease, respectively, during feeding.

The atherosclerosis induced experimentally in the rabbit by prolonged cholesterol feeding is accompanied by hypercholesterolemia and hyperlipoproteinemia. Ultracentrifugal studies have demonstrated a pronounced increase in concentration of low density serum lipoproteins during cholesterol feeding (1). Whether this increase reflects an increase in concentration of some or all of the lipoproteins normally present in rabbit serum, or whether it reflects the synthesis of lipoproteins not present previously, cannot be answered from ultracentrifugal studies alone, since lipoproteins of very similar or even identical sedimentation characteristics need not be chemically identical. As an additional means of answering this question, we have chosen to compare the NH_2-terminal amino acids of the serum lipoproteins before and at various intervals during cholesterol feeding. Thus a quantitative difference in NH_2-terminal amino acid content is evidence for a quantitative difference in the lipoproteins. Knowledge of the terminal amino acids should indicate some of the possible metabolic interconversions in an animal which is used extensively in studies of experimental atherosclerosis.

EXPERIMENTAL

New Zealand white female rabbits were fed Purina rabbit pellets containing 1 per cent cholesterol. The cholesterol was dissolved in ether, the solution was added slowly to the pellets with mixing, and the solvent was allowed to evaporate. For isolation of lipoproteins, 10 to 30 ml. blood were drawn from the ear vein.

Ultracentrifugal Isolation and Analysis. Lipoproteins were isolated from rabbit serum by centrifugation in solutions of varying density (2). The d $<$ 1.007 fraction[1] contained lipoproteins of hydrated density less than 1.007 g. per ml., and the d $=$ 1.007 to 1.019, 1.019 to 1.065, and 1.065 to 1.20 g. per ml. fractions contained those of hydrated densities between the relevant solvent density values. The fractions were characterized by the density of the medium used in isolating them and by analysis in the Model E Spinco analytical ultracentrifuge. Weight average flotation coefficients were obtained by dividing the schlieren pattern into narrow strips, determining the concentration (c_i) and flotation coefficient (S_{f_i}) of each strip in the customary manner, and evaluating the quotient $\sum_i S_{f_i} c_i / \sum_i c_i$. The flotation coefficients were not corrected to zero concentration, nor were Johnston-Ogston corrections applied. However, by means of a Brice-Phoenix differential refractometer, and with the assumption that the specific refractive increment of the low density, d $<$ 1.065 lipoproteins was identical with that (0.154 ml. per g.) of the d $<$ 1.065 lipo-

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[1] Abbreviations used: d, density; S_f, flotation coefficient, the negative of the sedimentation coefficient in Svedberg units of species undergoing centripetal migration in a sodium chloride solution of density 1.0630 g./ml. at 26°C; and DNP, dinitrophenyl.

proteins of human serum in a NaCl solution of density 1.0630 g. per ml. at 26°C, the lipoprotein concentration of identical density fractions obtained during the experiment was kept constant for ultracentrifugal analysis. Thus, for purposes of relative comparison, a comparison of successive S_f values should be almost as meaningful as one of S_f^o values. The d < 1.007, 1.007 to 1.019, 1.019 to 1.065, and 1.065 to 1.20 g. per ml. fractions contained the S_f 20 and greater, S_f 12-20, S_f 0-12, and high density lipoproteins of rabbit serum, respectively.

Terminal Amino Acid Analysis. NH_2-terminal amino acid analysis by the dinitrofluorobenzene technique (3) was carried out with the same general procedures used previously with human serum lipoproteins (2). The only differences in technique were the use of a 180-minute reaction period at room temperature for the mixture of 1 volume aqueous lipoprotein solution containing 1 per cent $NaHCO_3$ and 2 volumes of 2.5 per cent 2,4-dinitrofluorobenzene in ethanol, and the use of a 16-hour hydrolysis at 105°C with the redistilled HCl. After reaction with the dinitrofluorobenzene, the DNP-lipoprotein was washed with water and with combinations of ethyl ether, ethanol, methanol, acetone, methylal, and chloroform. Corrections for loss of NH_2-terminal amino acids during hydrolysis and subsequent procedures were made with the assumption that their recoveries from the protein were the same as those for the corresponding free DNP-amino acids under the same experimental conditions. A known amount of the relevant DNP-amino acid[2] was added to a weighed sample of DNP-protein. Hydrolysis, extraction, chromatography, and measurement of the optical density of the chromatogram eluate were carried out simultaneously with a weighed sample of DNP-protein without added DNP-amino acid. Subtraction of the amount of DNP-amino acid derived from the protein gave recoveries of 70 to 73, 76, and 65 to 68 per cent, respectively, for the added DNP-aspartic acid, DNP-glutamic acid, and DNP-serine. Essentially the same recoveries were obtained for these DNP-amino acids analyzed in the absence of protein. Added DNP-glutamic acid did not alter the yield of DNP-aspartic acid from DNP-protein, and added DNP-aspartic acid did not alter the yield of DNP-glutamic acid.

RESULTS AND DISCUSSION

Ultracentrifugal Analysis. The sedimentation coefficients of the lipoproteins during a representative experiment are summarized in Table 1. As observed by others (1), both the S_f rate and concentration of the d < 1.065 lipoproteins increased monotonically with continued feeding. The most pronounced increase in concentration occurred in the lipoproteins with S_f values of 20 or more, with serum levels of 1500 mg. per 100 ml. not uncommon after 12 weeks. After this period of feeding, the S_f 0-12 levels were about 200 mg. per 100 ml. Aortal plaques characteristic of cholesterol-fed rabbits appeared in most of the animals after 4½ weeks of feeding. None of the aortas were spared the plaques after 8 weeks, indicating that experimental atherosclerosis had certainly been achieved by this time. Plaques were still present in the aortas of rabbits fed a normal diet for 24 weeks after 16 weeks of cholesterol feeding. Low density serum lipoprotein levels in these rabbits had returned to values 10 to 20 mg. per 100 ml. above those (< 40 mg. per 100 ml.) before feeding.

Terminal Amino Acid Analysis. A study of terminal amino acid changes in the lipoproteins of a single animal during the feeding period would have been complicated by the increase in concentration of low density lipoproteins that follows extensive blood loss (4). Neither the differences in data obtained on lipoproteins from pools of 3, 5, and 7 rabbit sera nor that between duplicate analyses of the same sample was greater than 10 per cent. With animals fed cholesterol for 9 or for 15 weeks, 80 ml. blood yielded enough material for analysis of the d < 1.007, 1.019 to 1.065, and 1.065 to 1.20 lipoprotein fractions.[3] The differences were less than 10 per cent between these data from a single animal and those on similar lipoproteins from pooled blood of 3 and 7 rabbits fed cholesterol for 9 and 15 weeks. It is thus unlikely that the significance of the values of Table 1 was made uncertain by the necessity of using pooled blood.

The NH_2-terminal amino acid composition of the lipoproteins of rabbit serum before and during cholesterol feeding, and 24 weeks after the end of feeding and restoration to a normal diet, is summarized in Table 1. In addition to the NH_2-terminal amino acids given in Table 1, smaller amounts (1.0 to 2.5 $\times$ 10^6 g. protein per mole serine) of DNP-serine were found in all the lipoprotein fractions. Since these amino acids occur in phospholipids, one must be concerned whether the extensive washings with the previously mentioned combinations of organic solvents were effective in removing phospholipids from the DNP-protein before

[2] Mann Research Biochemicals, New York, N. Y.

[3] These rabbits were removed from the experiment after the bleeding.

TABLE 1. NH_2-TERMINAL AMINO ACIDS OF THE SERUM LIPOPROTEINS OF NORMAL AND HYPERLIPOPROTEINEMIC RABBITS

Density	S_f	Weeks on Diet*	G. Protein/Mole NH_2-Terminal Amino Acid	
			Aspartic Acid	Glutamic Acid
g./ml.				
<1.007	29	0	6.7×10^4	2.7×10^5
	40	4½	8.2×10^4	3.6×10^5
	51	9	7.5×10^4	3.6×10^5
	67	15	8.2×10^4	2.9×10^5
	32	15†	7.1×10^4	2.7×10^5
1.007–1.019	15.0	4½	1.7×10^5	5.6×10^5
	17.2	9	1.7×10^5	4.6×10^5
	18.7	15	1.8×10^5	4.8×10^5
1.019–1.065	6.4	0	2.1×10^5	7.4×10^5
	7.3	4½	2.4×10^5	5.4×10^5
	9.3	9	2.6×10^5	4.2×10^5
	10.2	15	2.6×10^5	3.9×10^5
	6.2	15†	2.2×10^5	7.6×10^5
1.065–1.20		0	4.0×10^4	
		9	3.9×10^4	
		15	4.2×10^4	2.0×10^6

* Rabbit pellets containing 1% cholesterol.

† These lipoproteins were obtained from animals kept on rabbit pellets 24 weeks after having been fed the cholesterol diet 15 weeks.

hydrolysis. The following experiment suggests that phospholipids, except possibly tightly bound ones, were effectively removed.

Male rats were injected with 200 μc. P^{32} orthophosphate. Sixteen hours later their livers were removed, washed with isotonic saline, and extracted with methylal, methanol, chloroform, and ethyl ether. The lipid extract was then fractionated on silicic acid columns (5) and 99.8 per cent of the radioactivity added to the column was found in the methanol eluate. Infrared spectrophotometry (5) confirmed the phospholipid nature of the eluate. An amount of phospholipid corresponding to 25 per cent by weight of the lipoprotein fraction was added to $d < 1.007$ and $d = 1.019$ to 1.065 lipoproteins of known terminal amino acid content, and the mixture was reacted with 2,4-dinitrofluorobenzene. The P^{32} content of the DNP-protein after washing, which was less than 0.08 per cent of the radioactivity associated with the insoluble lipoprotein after reaction with dinitrofluorobenzene, and the subsequent quantitative terminal amino acid determinations indicated that phospholipid contaminant could have accounted for 20 to 40 per cent of the NH_2-terminal serine, but for only a minor (less than 10 per cent) amount of the terminal aspartic or glutamic acids found.

These data support the peptide origin of at least aspartic and glutamic acids. Delipidation of rabbit lipoproteins prior to reaction with dinitrofluorobenzene was not done in this study. However, quantitative NH_2-terminal amino acid determination of S_f 6-8 and high density human serum lipoproteins was not affected by delipidation prior or subsequent to reaction with dinitrofluorobenzene.[4] Nevertheless, it can be argued that until the lipoproteins have been separated into discrete NH_2-terminal aspartic acid, glutamic acid, and serine containing lipoproteins, and NH_2-terminal peptide fragments containing the relevant amino acid have been isolated from the lipoproteins, definite conclusions regarding the peptide origin of all the NH_2-terminal amino acids found and the lengths of the peptide chains from which they came cannot be made. Reasonable estimates of lipoprotein molecular weights and protein content give values for the molecular weights of the protein moieties which are less than the values of g. protein per mole NH_2-terminal serine, except possibly in the $d < 1.007$ and 1.007 to 1.019 lipoproteins. The serine found could possibly represent a lipoprotein or lipoproteins present in relatively low concentration, a contaminating protein, or phospholipid.

The presence of a common protein component in the rabbit lipoproteins, as suggested by the common NH_2-aspartic acid, would be compatible with metabolic interconversions not of a proteolytic nature. Thus one lipoprotein could be converted to another by processes which remove lipids, such as the action of lipases or phospholipases or the transfer of lipids to other protein or lipoprotein acceptors. As may be seen from Table 1, considerable variation in the g. protein per mole NH_2-aspartic acid exists among the various lipoproteins. The variation in the amount of NH_2-aspartic acid in the protein components of the low density lipoproteins might be attributable to the presence in varying amounts of an NH_2-glutamic acid chain. However, since the $d < 1.007$ protein has greater percentages of both NH_2-terminal aspartic and glutamic acids than are found in the 1.007 to 1.019 or 1.019 to 1.065 proteins, this seems unlikely. Our data thus seem to indicate that two or more peptide chains of different molecular weight and the same end group are present

[4] B. Shore and V. Shore, unpublished data.

in these lipoproteins. In short, the data, while not eliminating the presence of a common protein chain or chains in the lipoproteins, do not give positive support for their existence.

The occurrence after cholesterol feeding of an NH_2-terminal amino acid not present before feeding would of course have been good evidence for the synthesis of a new lipoprotein. As may be seen from Table 1, aspartic acid, glutamic acid, and serine were the only NH_2-terminal amino acids found throughout the entire experimental period.

The data of Table 1 indicate that the quantitative NH_2-terminal aspartic acid content of the $d = 1.065$ to 1.20 high density lipoproteins of rabbit serum does not change throughout the cholesterol feeding. If we assume that only one protein chain is present, then the minimum molecular weights of the NH_2-terminal aspartic acid containing proteins of the $d = 1.065$ to 1.20 lipoproteins of three species are not greatly different: 40,000[5] or 54,000 (6) in man, 46,000 (7) in the dog, and 40,000 in the rabbit. Table 2 presents the comparative biochemistry of the NH_2-terminal amino acids of the serum lipoproteins of various species. In contrast to the other species, aspartic acid is the major NH_2-terminal amino acid of all of the lipoproteins of rabbit serum.

TABLE 2. NH_2-TERMINAL AMINO ACIDS OF SERUM LIPOPROTEINS OF VARIOUS SPECIES

Density of Lipoprotein	Species*	NH_2-Terminal Amino Acids	
		Principal Components	Minor Components†
g./ml.			
<1.007	Man (2, 6, 9)	Serine, threonine, aspartic acid	Glutamic acid
	Rabbit	Aspartic acid	Glutamic acid, serine
1.007–1.019	Man (6)	Serine, threonine, aspartic acid, glutamic acid‡	
	Rabbit	Aspartic acid, glutamic acid	Serine
1.019–1.065	Man (2, 6, 8, 9)	Glutamic acid	Serine, threonine, aspartic acid
	Rabbit	Aspartic acid	Glutamic acid
	Dog (7)	Glutamic acid	
1.065–1.20	Man (2, 6, 8, 9, 10)	Aspartic acid	Serine, threonine
	Rabbit	Aspartic acid	
	Dog (7)	Aspartic acid	

* Numbers in parentheses refer to References.

† Refers to NH_2-terminal amino acids which were less than 20 per cent of the total moles of amino acid recovered from the protein analyzed.

‡ Data from Reference 6 and our unpublished data.

Because the terminal amino acid composition of the $d < 1.007$ lipoproteins fluctuated irregularly rather than monotonically, it is difficult and perhaps unwise to attempt to attach any significance to these data. Any variation in NH_2-terminal groups may be merely a reflection of a variation in the relative amounts of the lipoproteins comprising this spectrum of molecules. Indeed, the ultracentrifugal data indicate a shift to higher S_f values and a tremendous increase in concentration in this heterogeneous fraction following cholesterol feeding. Insufficient $d = 1.007$ to 1.019 lipoproteins from animals fed no cholesterol was available for comparison, but there were no quantitative differences in terminal amino acids among samples 4½, 9, and 15 weeks after feeding.

During cholesterol feeding there were consistently monotonic increases in NH_2-glutamic acid composition and decreases in NH_2-aspartic acid composition of the $d = 1.019$ to 1.065 lipoproteins. As Table 1 indicates, after the rabbits were returned to a normal diet for 24 weeks after the 15 weeks on cholesterol, the relative proportion in their 1.019 to 1.065 lipoproteins of the NH_2-terminal aspartic and glutamic acid lipoproteins, which had been changing throughout the feeding period, became that before feeding.

It is clear that comparison of ultracentrifugal patterns and NH_2-terminal amino acid content is a first step in distinguishing among closely related lipoproteins. It seems necessary to supplement these data with immunochemical studies, with proteolysis and two-dimensional chromatography-electrophoresis of the resultant peptides (7, 11), and with other chemical and physical procedures. These techniques should also be valuable in deciding whether any lipoprotein fractions of differing density have a common NH_2-terminal aspartic acid protein chain.

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