Structural Investigation of the Low-Density Lipoprotein of Hen's Egg Yolk Using Proteolysis*

D. C. Steer,† W. G. Martin, and W. H. Cook

ABSTRACT: To obtain information on the location of the protein moiety in low-density lipoproteins, comparative proteolysis studies using trypsin and Pronase were carried out on the low-density lipoprotein of hen's egg yolk and on its partially and completely delipidated forms. The results demonstrated that 20% of the protein of the native lipoprotein was not accessible to Pronase, although the resistant portion consisted of peptides of considerable size. In contrast, the protein of the partially and completely delipidated forms was completely digested by Pronase. The most likely explanation was considered to be that the resistant peptide is not at the surface in native lipoprotein particles, and three possible models were proposed. An increase in particle size was observed to follow proteolysis of the lipoprotein, and the resulting product was stable and freely water soluble. It was proposed that the increase in size changed the surface-to-volume ratio to a level at which phospholipid and possibly residual protein could give the degree of surface coverage existing in the native lipoprotein. Values were calculated for the surface area available per phospholipid molecule, assuming that the surface of the Pronase-treated particle is covered either by phospholipid alone or by phospholipid plus cholesterol. The results were 70 and 56 Å^2, respectively, and as the second value is in better agreement with X-ray data, it was concluded that material other than phospholipid is probably also present at the surface of Pronase-treated low-density lipoprotein fraction. Using the value of 56 Å^2/phospholipid, 16.7 Å^2/amino acid residue was calculated for the enzyme-accessible protein of the native lipoprotein, which seems to be consistent with results for polypeptide monolayers.

Little is known about the location or conformation of the protein moiety of low-density lipoproteins. Earlier authors have commonly considered the protein to be at the surface although in the fully extended form the amount of protein present would cover less than two-thirds of the surface of most low-density lipoproteins (Cook and Martin, 1962). However, Margolis and Langdon (1966) have shown that the protein in β-lipoprotein is not completely hydrolyzed by proteolytic enzymes and hence is either not entirely at the surface or is protected by other means, *e.g.*, lipid–protein complexes.

The low-density lipoprotein fraction of egg yolk containing 10–14% protein, 20–25% phospholipid, and 60–65% neutral lipid is a favorable material for extending this kind of examination. In addition to native low-density fraction and the delipidated protein (vitellenin) low-density fraction can be partially delipidated to produce soluble subunits that have been characterized as to lipid content and other properties (Augustyniak *et al.*, 1964). The size and lipid content (*ca.* 50% for the most abundant subunit) of these indicate that they probably do not have a lipid core structure (Cook and Martin, 1962). Native low-density fraction has two components, low-density fraction 1 and low-density fraction 2 (Martin *et al.*, 1964), both of which are polydisperse with average M = 10 and 3 × 10^6, respectively. On partial delipidation, both yield subunits which are indistinguishable on the basis of size and lipid content (Augustyniak *et al.*, 1964).

In the present investigation comparative studies on the proteolysis of low-density fraction were made at all three lipid levels (native, partially delipidated, and completely delipidated) to obtain information concerning the disposition of the protein in the native lipoprotein.

Experimental Section

Materials. Trypsin (once crystallized) was purchased from Mann Research Laboratories Inc. and Pronase (B grade) from Calbiochem.

Low-density fraction was prepared according to Martin *et al.* (1963) except that for the initial centrifugation yolk was diluted with an equal volume of a solution of 0.1 M NaCl, 0.04 M NaF, and 0.04% EDTA.

Partial lipid extraction was performed by a modification of the procedure of Augustyniak *et al.* (1964). Solutions of 5–10% low-density fraction in 0.2 M NaCl were stirred with 20 volumes of ether saturated with the same solvent, in a conical flask at 3–5°. A magnetic stirrer was used, and the rate was adjusted to prevent droplets of the aqueous phase from reaching the upper surface of the ether during extraction. Ether was changed twice during the 3-hr extraction period. After extraction the aqueous phase was transferred to a shallow dish and dissolved ether was removed under slightly reduced pressure over concentrated H_2SO_4. The resul-

* From the Division of Biosciences, National Research Council, Ottawa, Canada. *Received April 22, 1968.*

† National Research Council Postdoctorate Fellow, 1966–1968.

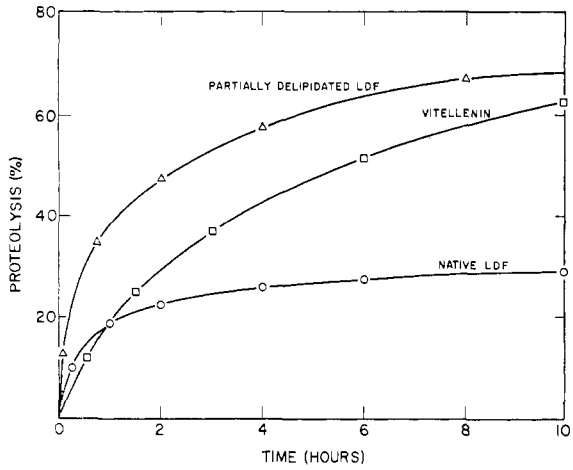

FIGURE 1: Rate of tryptic hydrolysis of low-density fraction and derivatives.

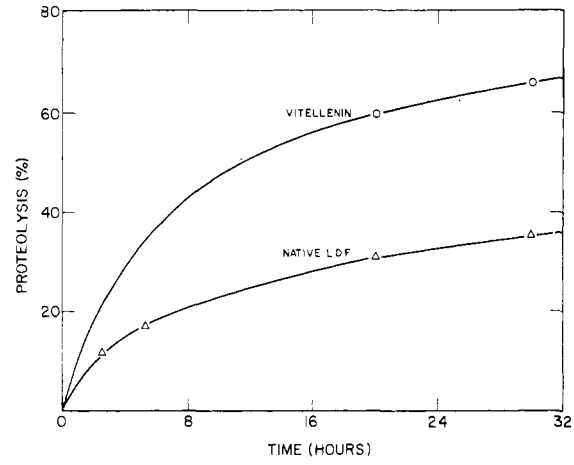

FIGURE 2: Rate of hydrolysis of low-density fraction and vitellenin by Pronase.

tant solution was centrifuged for 1 hr at 78,000*g* to remove insoluble material.

Lipid-free protein (vitellenin) was prepared by extracting low-density fraction with 1-butanol at 4°. The protein precipitate was collected by centrifugation, washed several times with ether to remove all traces of butanol, and dried *in vacuo*.

Estimation of Extent of Proteolysis. Tryptic hydrolysis was carried out at 35° and pH 8.4 under nitrogen and monitored with a pH-Stat (Radiometer Ltd.). The enzyme concentration was 1% of the substrate protein concentration. Extent of proteolysis was calculated as described by Margolis and Langdon (1966).

Hydrolysis by Pronase was determined using the ninhydrin reaction. Solutions or suspensions of substrate in 0.1 M $NaHCO_3$, 0.02 M NaF, and 0.2 M NaCl were incubated at 2% enzyme-to-substrate protein ratio (corrected for extraneous material in the enzyme preparation). Aliquots were removed at suitable time intervals and precipitated with two volumes of 0.3 M trichloroacetic acid and the ninhydrin procedure of Moore and Stein (1948) was carried out on the soluble fraction. The color yield was compared with that for solutions obtained by the hydrolysis of vitellenin with 6 N HCl at 110° for 20 hr.

The extent of proteolysis after Pronase digestion was also estimated by measuring the optical density of the trichloroacetic acid soluble fraction at 280 mμ (in 0.1 M NaOH). With some tryptic digests the trichloroacetic acid soluble fraction was analyzed by the procedure of Folin and Ciocalteu (1927). In each case, the optical density of the complete enzyme digest of vitellenin was measured under the same conditions and the per cent proteolysis calculated by comparing with the optical density of the trichloroacetic acid soluble fraction.

Another estimate of proteolysis was obtained from the peptide recovery determined from total nitrogen values, as described in the Results section.

Preparation of Pronase-Treated Low-Density Fraction and Isolation of the Residual Peptide. Large-scale Pronase digests of low-density fraction were prepared using 20–25% solutions of the lipoprotein. After 30-hr incubation the salt concentration was increased to 10% by the addition of solid NaCl, followed by centrifugation at 78,000*g* for 20 hr at 5°. The clear semisolid supernatant was collected, redispersed in 10% NaCl, and recentrifuged. The enzyme-modified low-density fraction was then dispersed in distilled water and dialyzed against 0.1 M NaCl under nitrogen. Residual peptide was prepared from this material by lyophilization followed by extraction with chloroform–methanol (3:2). The insoluble peptide was collected by centrifugation, washed with ethanol and water, and lyophilized.

Chemical and Physical Methods. Total nitrogen was estimated by the Kjeldahl method according to Mackenzie and Wallace (1954). Phosphorus was determined by the method of Ames (1966). Dry weights were obtained by drying dialyzed lipoprotein samples *in vacuo* at 60°. The contribution of salt to the dry weight of lipoprotein was obtained from the dry weight of the diffusate and appropriate corrections for salt displacement were applied (Martin *et al.*, 1964).

Solubilization of the residual peptide was accomplished by succinylation as described by Habeeb *et al.* (1958). Amino acid analyses were carried out using a Beckman Model 120 analyzer. Corrections for destruction of serine, threonine, and tyrosine were obtained by hydrolyzing samples for 20-, 45-, and 70-hr periods and extrapolating to zero time.

Partial specific volumes were obtained with a magnetic float apparatus as described by MacInnes and Dayhoff (1952). Light-scattering measurements at 436 mμ (BS light-scattering photometer, Phoenix Precision Instrument Co., Philadelphia) were corrected for dissymmetry of scatter. Refractive index increments were measured with a Brice-Phoenix differential refractometer. A Spinco Model E ultracentrifuge with schlieren optics was used for sedimentation and flotation experiments. The patterns obtained during flotation were asymmetrical and it was necessary to use the square root of the second moment of the concentration gradient curve for locating the boundary position, as proposed by Goldberg (1953). This was achieved by enlarging the patterns and making measurements with an Amsler mechanical integrator.

TABLE I: Survey of Extent of Proteolysis of Low-Density Fraction and Derivatives Indicated by Different Methods.

Enzyme	Digestion Period (hr)	Method	Extent of Proteolysis (%)[a]		
			Native	Partially Delipidated	Delipidated
Trypsin	10	pH-Stat	29 ± 0.9 (4)	68 ± 1.0 (2)	74 ± 1.9[b] (3)
Trypsin	24	Folin–Ciocalteu	67	93	89
Pronase	30	Ninhydrin	35		65
Pronase	24	Optical density at 280 mμ	77	95	96 ± 1.9 (3)

[a] Where more than one experiment was performed the number is given in parentheses, and the value given is the mean, with its estimated standard error. [b] This value only for a 20-hr digestion period.

TABLE II: Peptide Recovery after Proteolysis.

State of Lipoprotein	Enzyme	Digestion Period (hr)	Method of Removal of Digestive Products	Recovery[c] (%)
Native	Trypsin	10	Centrifugation[a] and dialysis	65.5 ± 2.1 (3)
Partially delipidated	Trypsin	10	Centrifugation[b] and dialysis	37 (1)
Delipidated	Trypsin	24	Dialysis	54 (1)
Delipidated	Trypsin	24	Precipitation with trichloroacetic acid	24 (1)
Native	Pronase	30	Centrifugation[a] and dialysis	22.8 ± 0.8 (3)
Partially delipidated	Pronase	24	Dialysis	2.5 (1)
Delipidated	Pronase	24	Dialysis	4.5 (1)
Delipidated	Pronase	24	Precipitation with trichloroacetic acid	4.6 (1)

[a] 78,000*g* in 10% NaCl. [b] 134,000*g* in NaBr (density = 1.17). [c] The number of experiments is given in parentheses; where more than one experiment was performed, the mean is given with the estimated standard error of the mean.

Results

Proteolysis. The time course of tryptic digestion of low-density fraction, the partially delipidated subunits, and vitellenin is shown in Figure 1, and for Pronase digestion of low-density fraction and vitellenin in Figure 2. Vitellenin, being insoluble, was digested as a suspension, and this probably accounts for its low initial rate of digestion. Tryptic digestion of low-density fraction tended to level off after 3–4-hr incubation, but Pronase digestion continued for a longer period and may not have been complete after 30 hr, the maximum period studied. Addition of more enzyme at later stages of digestion did not accelerate or increase hydrolysis.

A survey of the extent of proteolysis indicated by several methods is given in Table I. Percentage proteolysis figures indicated by different methods are not directly comparable because of the different criteria employed. Thus, tryptic digestion monitored with the pH-Stat was calculated as the percentage hydrolysis of bonds involving the carboxyl groups of arginine and lysine. In contrast, hydrolysis by Pronase determined with the ninhydrin method is given as the proportion of the total number of peptide bonds hydrolyzed, and these results will be subject to a low bias due to resistance of certain peptide bonds to Pronase and to the reduced color yield with ninhydrin given by peptides as opposed to free amino acids. In spite of these limitations, the results show consistently that under comparable conditions native low-density fraction was hydrolyzed to a lesser degree than its partially delipidated subunits or vitellenin. Since the extent of proteolysis with the last two forms was similar, it appears that any lipid–protein complexes present in the partially delipidated material did not affect the susceptibility to proteolysis of the protein moiety.

The amount of peptide remaining after proteolysis, for low-density fraction and partially delipidated low-density fraction (as lipopeptide) and for vitellenin (as nondialyzable or trichloroacetic acid insoluble peptide), is shown in Table II. Where centrifugation was employed for separation of the digestion products, the

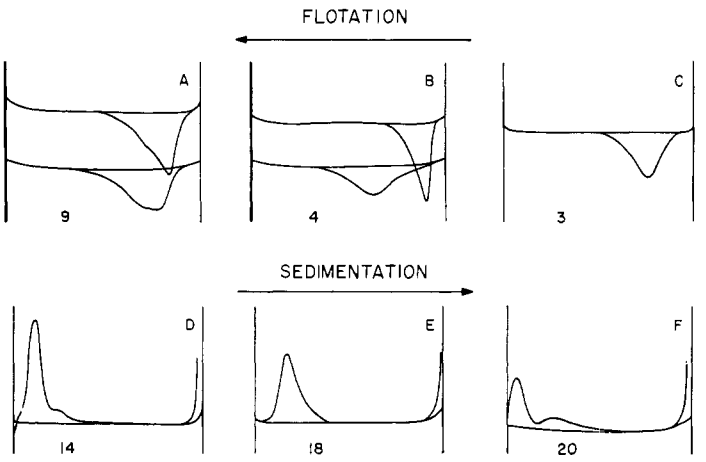

FIGURE 3: Ultracentrifugal schlieren patterns of low-density fraction and derivatives, before and after enzyme digestion. (A) Upper: low-density fraction; lower: 10-hr trypsin-treated low-density fraction (solvent 1 M NaCl). (B) Upper: low-density fraction; lower: 20-hr Pronase-treated low-density fraction (solvent 1 M NaCl). (C) 30-hr Pronase-treated low-density fraction (solvent 0.1 M NaCl). (D) Partially delipidated low-density fraction (solvent 0.12 M NaCl). (E) 24-hr Pronase-treated partially delipidated low-density fraction (solvent 0.12 M NaCl). (F) Succinylated residual peptide (solvent 0.2 M NaCl). Speed A–C, 42,040 rpm; D and E, 59,780 rpm; and F, 67,770 rpm. Elapsed time in minutes after reaching speed is indicated on the figure.

residual lipopeptide was recovered as a semisolid supernatant, the enzyme and low molecular weight digestion products remaining in the subnatant. Peptide recovery values were calculated from the nitrogen content of the lipoprotein before and after digestion, assuming that no phospholipid nitrogen was released during recovery. Although some phospholipid may be lost (see later and Table III) this would not be sufficient to affect the recoveries appreciably. For vitellenin, peptide recovery was calculated from the nitrogen content of the dialyzed digest or trichloroacetic acid precipitate, an allowance being made for enzyme nitrogen. With partially delipidated low-density fraction after Pronase digestion, nitrogen and phosphorus determinations were made, and peptide recoveries estimated from the protein to phospholipid ratio of the original solution and of the resultant digest after dialysis. These calculations involved the following data: total nitrogen of vitellenin = 14.5% (Cook *et al.*, 1962); mean molecular weight of phospholipid of low-density fraction = 785; and N/P ratio of the phospholipid = 1. Control experiments showed no detectable contribution of phosphorus from the protein moiety.

The results in Table II show the same over-all trend as those in Table I, the polypeptide residue recovered from low-density fraction being substantially greater than that from partly or completely delipidated material. There was a marked difference between the peptide recovery values for vitellenin after trypsin digestion, depending upon the method used for separation of the digestion products. This may be due to some relatively high molecular weight material recoverable as nondialyzable peptide, but not as trichloroacetic acid insoluble peptide. The large oligosaccharide group of vitellenin (mol wt *ca.* 1700; Augustyniak and Martin, 1968) could be responsible for the solubility of this fraction in trichloroacetic acid. Lengthy digestion with Pronase reduced the amount of polypeptide in low-density fraction to about 23% of the initial value and of the other substrates to less than 5%. It appears therefore that some 20% of the protein of low-density fraction is protected from complete hydrolysis, a figure that is consistent with the results in Table I.

TABLE III: Comparative Chemical and Physical Properties of Low-Density Fraction and Pronase-Treated Low-Density Fraction.

	Low-Density Fraction	Pronase-Treated Low-Density Fraction
Phosphorus (%)	0.87	0.91
Nitrogen (%)	2.05	0.81
Phospholipid (%)	22.0	23.0
Neutral lipid (%)	66.5	74.2
Protein (%)	11.4	2.73
dn/dc (436 mμ)	0.158	0.151
$\bar{v}$ (in 0.1 N NaCl)	1.026	1.053 (1.053)[a]
$s^0_{20,w}$ (S)	−12.7	−71.6 (−64.2)[a]
Molecular weight from $s^0_{20,w}$[b]	9.3×10^6	41×10^6
Molecular weight from light scattering (g)	12.6×10^6	52×10^6

[a] An independent preparation had the values in parentheses. [b] Assuming a spherical shape.

Ultracentrifugal Studies. Flotation patterns of native, trypsin, and Pronase-treated low-density fraction are shown in Figure 3A–C. Tryptic hydrolysis reduced the proportion of material rising at the rate of low-density fraction 2 in untreated lipoprotein preparations with a concomitant increase in amount of the faster floating material (low-density fraction 1, in native low-density fraction). After 20-hr Pronase digestion, the only evidence of material with the same flotation rate as the original low-density fraction was a small trailing shoulder which was absent after a 30-hr digestion period (Figure 3C). The Pronase-treated low-density fraction had a greatly increased flotation rate indicative of the expected increase in apparent specific volume, and also (as demonstrated by the data in Table III) of a substantial increase in particle size. Figure 3A shows that trypsin digestion produces the same effect but to a lesser degree.

As shown in Figure 3D the extraction procedure used to produce the partially delipidated subunits yielded largely the 4S component of Augustyniak *et al.* (1964). Following 24-hr digestion with Pronase the sedimentation rate of this component was 6.3 S in 0.12 M NaCl (Figure 3E). Considering that 95% of the protein had been removed with a concomitant increase in apparent

specific volume, this increase in rate represents a substantial increase in particle size.

Complete delipidation of Pronase-treated low-density fraction yielded an insoluble polypeptide fraction of which more than 80% could be solubilized by succinylation. The ultracentrifuge pattern (Figure 3F) for this material shows a major peak having $s^0_{20,w}$ = 1.7 S, compared with $s^0_{20,w}$ values of 3.3 S for succinylated vitellenin in 0.2 N NaCl (W. G. Martin, unpublished data) and 2.4–2.8 S for vitellenin dissolved in formic acid (Martin, 1961). The minor fast-sedimenting boundary may be aggregated material as this tendency has also been observed in the parent protein (Martin *et al.*, 1959). These results indicate that while a polypeptide of substantial size survives Pronase digestion of low-density fraction, it is smaller than the estimated size of the protein of native low-density fraction.

Chemical and Physical Properties of Pronase-Treated Low-Density Fraction. A preparation of low-density fraction was treated with Pronase for 30 hr and the residual material was recovered by centrifugation and dialysis. A portion of the same preparation of low-density fraction was taken through the same manipulations without enzyme to provide a basis for comparison. The results of physical and chemical measurements together with some calculated quantities are summarized in Table III. Phospholipid, neutral lipid, and protein were calculated from the N and P values with the same assumptions used in the determination of peptide recovery.

Manipulations of solutions of low-density fraction involve some loss of lipoprotein; thus it was not possible to determine directly the recovery of lipoprotein after enzyme digestion. However, if it is assumed that none of the neutral lipid was lost selectively, then from the data in Table III it can be calculated that 21.4% of the original protein and 93.7% of the phospholipid remained after Pronase digestion.

The apparent partial specific volume, $\bar{v}$, increased on Pronase treatment as would be expected from protein loss. From $\bar{v}$ (1.026) of native low-density fraction the $\bar{v}$ of Pronase-treated low-density lipoprotein fraction can be calculated on the basis of protein removed ($\bar{v}$ = 0.742; Martin *et al.*, 1959). The value obtained was 1.054, in good agreement with the observed value of 1.053, a result that provides confirmation of the recovery figures reported.

The flotation rate reported here for low-density fraction is considerably higher than the value of 7.5 S found previously by Martin *et al.* (1959). This apparent difference arises largely from the use of a different method of locating the boundary. If, as previously, the maximum ordinate had been used instead of the square root of the second moment, the observed flotation rate would have been 8.0 S.

These considerations in no way invalidate the relative estimates of size for digested and native low-density fraction as given in Table III, and it is evident from these results that an approximate fourfold increase in mean particle size occurred after Pronase digestion.

Amino Acid Analysis. The amino acid compositions of vitellenin and of the polypeptide from Pronase-digested low-density fraction are given in Table IV. Some significant differences are indicated, the largest of which was in serine where the residual peptide contains about 3% more than vitellenin. Other amino acids which showed appreciable differences were aspartic acid and phenylalanine (increased in the residual peptide) and isoleucine, valine, and arginine (reduced). Taken as a group, amino acids with hydrophobic side chains show no marked change, being if anything present in smaller proportion in the residual peptide.

TABLE IV: Amino Acid Composition of Vitellenin and the Residual Peptide from Pronase-Treated Low-Density Fraction.

	Vitellenin (mole %)[a]	Residual Peptide (mole %)[b]
Asp	10.94 ± 0.17	13.09 ± 0.06
Thr	6.36 ± 0.06	7.01 ± 0.20
Ser	7.02 ± 0.01	10.45 ± 0.17
Glu	10.94 ± 0.33	9.49 ± 0.55
Pro	3.35 ± 0.14	2.38 ± 0.19
Gly	5.17 ± 0.01	5.83 ± 0.38
Ala	7.96 ± 0.10	7.09 ± 0.22
Val	6.91 ± 0.04	5.77 ± 0.12
Met	1.05 ± 0.19	1.57 ± 0.34
Ile	6.53 ± 0.01	4.69 ± 0.33
Leu	10.89 ± 0.14	10.85 ± 0.03
Tyr	3.90 ± 0.07	3.49 ± 0.29
Phe	3.98 ± 0.02	5.30 ± 0.13
Lys	7.59 ± 0.04	6.65 ± 0.36
His	1.30 ± 0.06	2.13 ± 0.21
Arg	5.36 ± 0.04	3.81 ± 0.13
Half-cystine	0.80 ± 0.08	0.41 ± 0.02
Glucosamine[c]	0.36	0.54

[a] Mean of two independent preparations with estimated standard error of mean. [b] Mean of three independent preparations with estimated standard error of mean. [c] As moles of glucosamine/100 moles of amino acids after 20-hr hydrolysis not corrected for destruction.

Discussion

While there was some discrepancy between methods as to the extent of proteolysis, presumably due to systematic bias, they all agree that lipid-free vitellenin and partially delipidated low-density fraction were digested to a greater extent than native low-density fraction. The peptide recovery values are also consistent with these results and probably represent the least biased estimate of the extent of proteolysis. Evidently about 20% of the protein of native low-density fraction is inaccessible to proteolytic enzymes, but after partial or complete delipidation, it becomes accessible. This is in agreement with the findings of Margolis and Langdon (1966) who compared the extent of proteolysis of native β-lipoprotein and its lipid-free protein. As stated by these authors,

such results do not establish unequivocally that part of the protein is "buried" in the interior of the particle, since the presence of lipid could inhibit proteolytic action in other ways. While the lipid composition of native and partially delipidated low-density fraction is obviously different, the present study shows that the presence of lipid *per se* has little or no inhibitory effect on proteolysis.

Ultracentrifugal studies indicated that a peptide of considerable size survived Pronase digestion of low-density fraction. Particles of low-density fraction each contain several protein molecules and estimates of their number and size are variable because of their limited solubility and tendency to aggregate following delipidation (Martin, 1961). However, the size of the residual peptide after Pronase digestion indicated by the sedimentation rate of the succinylated peptide appears to be smaller than the minimum size of the protein molecules in native low-density fraction. From this it appears that all protein molecules are attacked by Pronase and some may be completely digested.

In native low-density fraction the residual peptide is evidently not accessible to Pronase, although this enzyme has a wide range of specificity (Nomoto *et al.*, 1960) and is capable of completely digesting the partially or completely delipidated protein. The peptide could be at the surface as a lipid–protein complex that resists attack unless altered by complete or partial delipidation. However, considering the size of the peptide and its amino acid composition, it seems unlikely that it would be completely protected throughout its entire length by lipid–protein complexes. Another possibility is that this peptide has a resistant conformation in native low-density fraction that is destroyed on removal of lipid. Again, it seems unlikely that such a conformation would survive Pronase digestion if it were entirely at the surface. If a surface location is ruled out, three other types of protection can be envisaged: the backbone of the resistant peptide is interdigitated between surface lipids; the peptide is located at the contact area between associated lipoprotein molecules; the peptide is buried within the neutral lipid core, a situation which requires some folding of the peptide to accommodate the relatively high proportion of polar groups revealed by amino acid analysis. The evidence does not permit a distinction between these possibilities.

Phospholipase C removes 95% of the lipid phosphorus of low-density fraction (Burley and Kushner, 1963); thus the phospholipid is probably at the surface. The fact that phospholipid is virtually insoluble in neutral lipid (Schneider and Tattrie, 1968) is additional evidence for this. Most of the protein is also at the surface as 80% can be digested by Pronase. When either of these components is destroyed, loss of stability occurs, as was indicated by a rapid increase in turbidity during digestion of low-density fraction by the lipase and by an increase in turbidity or particle size following the proteolysis of several lipoproteins (Banaszak and McDonald, 1962; Girard and Canal, 1965; Margolis and Langdon, 1966; Margolis, 1967). In the present study an increase in particle size was also observed following the proteolysis of both native and partially delipidated low-density fraction. This is considered to be the result of a process of aggregation, which must be limited in extent, since the material remains soluble.

Such behavior can be explained if a stable particle is obtained only when sufficient polar material is present to cover a critical proportion of the surface. Thus when part of the protein is removed by proteolysis, an increase in particle size occurs, reducing the surface-to-volume ratio to a level that will permit phospholipid to provide the required coverage. After Pronase digestion it appears that all surface protein is removed; thus the enzyme-modified particle will be covered only by phospholipid plus any cholesterol or neutral lipid associated with the surface layer. From the surface area of this particle, assuming all the phospholipid to be at the surface, it is possible to compute the surface area available per phospholipid molecule and to compare it with molecular areas for phospholipid observed by others.

The basic equation used was

$$S = n_p s_p \qquad (1)$$

where S = average surface area of particle, calculated assuming spherical shape from $S = (17.65M\bar{v})^{2/3}$; n_p = number of phospholipid molecules per particle computed from the average particle size and composition; and s_p = area available per phospholipid molecule.

A maximum value of 70 Å²/phospholipid is obtained when it is assumed that phospholipid only forms the surface of the particle. If all the cholesterol (4% of low-density fraction lipids; Martin *et al.*, 1963) was also at the surface, taking its molecular area as 35 Å² and reducing the total area S (eq 1) accordingly, the area available per phospholipid would be reduced to 56 Å². The X-ray data of Rand and Luzzati (1968) indicate 51 Å² for erythrocyte phospholipids in the presence of 26% cholesterol, but they note that cholesterol has a condensing effect on phospholipid packing. As the surface layer of Pronase-treated low-density fraction can contain at most 15% cholesterol, their figure of 51 Å² will be somewhat low for comparison with low-density fraction phospholipid. The computed value of 56 Å² therefore seems to be in reasonable agreement with the X-ray data, which means that the particle surface can accommodate material other than phospholipid. For the calculation this was assumed to be cholesterol, which is likely since cholesterol is normally associated with phospholipid in biological materials. However, as only part of the cholesterol may be at the surface, and due to the approximate nature of the calculation, the presence of small amounts of triglyceride at the surface cannot be ruled out, nor of part of the residual peptide could its resistance to Pronase be reconciled with a surface location.

Using the value of 56 Å²/phospholipid and again assuming that all the phospholipid and cholesterol are at the surface, one can calculate the area available to the enzyme-accessible protein in native low-density fraction. A figure of 16.7 Å²/amino acid residue (mean residue weight 112) is obtained which is consistent with the results reported by Malcolm (1965) who obtained 13.8

Å²/residue for monolayers of poly-D-alanine (residue weight 71). Had the enzyme-accessible protein in low-density fraction been present at the surface in a globular form, a much smaller area per residue would have been expected.

Previously it was shown that lipoproteins are of two types, low-protein lipoproteins, *e.g.*, low-density fraction, which have a neutral lipid core, and high-protein lipoproteins (Cook and Martin, 1962). From these results, it appears that the amount of phospholipid and surface protein is a determinant of particle size in low-density fraction, and probably in other low-protein lipoproteins.

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