Binding of Lipid to Protein in Lipovitellin from the Hen's Egg*

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ABSTRACT: Lipovitellin, isolated from fresh hen's eggs, was used to study the binding of lipid to protein in a high-density lipoprotein. The influence of various treatments and of enzymatic digestion of the lipovitellin on the amount of lipid extracted by ether was determined. Treatment of lipovitellin with urea, guanidine hydrochloride, sodium thioglycolate, or nonionic detergents did not increase the amount of lipid extracted with ether, but treatment with sodium deoxycholate or sodium dodecyl sulfate increased it from 17 to 30–45%. Digestion with phospholipase C or D did not increase the lipid extracted. Lipovitellin was digested sequentially with trypsin, papain, and Pronase. After each digestion, the lipids were extracted from the digest by ether, and soluble peptides were separated from the insoluble residue by centrifugation. Peptides were analyzed for amino acid content, and lipids were fractionated by chromatography through silicic acid. The compositions of the peptides and lipids released by trypsin from lipovitellin were different from those released by papain or Pronase from the residues.

The data obtained indicate that most of the lipid is held within the protein network of the lipovitellin molecule in such a way that it is not reached by ether during extraction. About 37% or less of the lipid is bound on or near the surface of the molecule where it can be extracted by ether. When part of the molecule is removed by proteolysis or the molecule is opened up by disruption of hydrophobic bonding, ether can then extract part of the previously unextractable lipids, and further proteolytic digestion exposes more lipid to ether extraction.

Little is known of the structure of lipoproteins. The low-density lipoproteins have been postulated as a core of lipid and water or of lipid surrounded by a thin film of protein and phospholipid and perhaps cholesterol (Gurd, 1960; Cook and Martin, 1962; Vandenheuvel, 1962). Lipid may be combined with protein in a different manner in high- and low-density lipoproteins. Lipovitellin, from hen's eggs, was used in the present investigation to study the binding of lipid to protein, because it is a high-density lipoprotein that can be isolated readily in a fairly reproducible condition from an easily available source and because its properties have been studied extensively (Cook, 1961).

Burley and Cook (1961) developed a procedure for isolating lipovitellin from egg yolk and of separating it from the low-density lipoproteins and phosvitin.

Proteolytic enzymes have been used by Ashworth and Green (1963) in their studies of human α-lipoprotein and by Banazak and McDonald (1962), by Bernfeld and Kelley (1964), and by Margolis and Langdon (1966) in studies of the β-lipoproteins. Glick (1963) studied the hydrolysis of lipovitellin by papain and separated the digested lipovitellin into two parts.

Lipovitellin is insoluble in water or dilute buffer solutions, and this property simplifies the separation of soluble peptides from the insoluble lipoprotein. Digestion with trypsin, a highly specific enzyme (Desnuelle, 1960), was used in the present study to remove part of the protein as soluble peptides. The insoluble residue was next hydrolyzed with papain, a proteinase of intermediate specificity (Smith and Kimmel, 1960), and the resulting residue was hydrolyzed with Pronase, a highly nonspecific proteinase (Nomoto *et al.*, 1960). Ether-soluble lipids were removed after each hydrolysis.

Experimental Section

Lipovitellin was prepared in two slightly different ways, both adaptations of the procedures of Burley and Cook (1961). Yolks of four eggs were diluted with an equal weight of 0.16 M sodium chloride solution, mixed well, and filtered through cheesecloth to remove the chalazae, vitelline membrane, and any adhering white. The yolk granules were separated from the supernatant low-density fraction and the water-soluble yolk proteins by centrifuging in an MSE 1700 or in a Sorvall RC-2 refrigerated centrifuge (0–2°) for 30 min at 17,000 rpm. The granules were dispersed in a volume of 0.16 M sodium chloride solution equal to twice that used to dilute the original yolk, by grinding in a porcelain mortar. The granules were again separated, in early studies, by centrifugation as before, dissolved in 50 ml of 5% sodium chloride solution, and dialyzed against 0.16 M sodium

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chloride solution at 2° to precipitate the lipovitellin and separate it from the low-density fraction, which is soluble in 0.16 M sodium chloride solution. This procedure was repeated, and the granules, after recovery by centrifugation, were dissolved in 50 ml, of 1.0 M sodium acetate solution by grinding in a mortar. Water (21 ml) was added to dilute to 0.7 M sodium acetate, 29 ml of 0.7 M sodium acetate was added to make the volume to 100 ml, and the solution was centrifuged for 30 min at 13,800 rpm to remove any undissolved material. In later studies the granules were dissolved in 60 ml of 1.0 M sodium acetate solution by grinding in a mortar, and the cloudy solution was centrifuged at 0° for 16 hr at 30,000 rpm in a Beckman Model L ultracentrifuge using the no. 30 rotor. The floating material was removed, and the subnatant was diluted to 0.7 M sodium acetate, made to a volume of 100 ml with 0.7 M sodium acetate solution, and centrifuged at 17,000 rpm for 30 min to remove undissolved material. The supernatant was then poured onto a column (2 or 2.5 × 65 cm) of Dowex 1-X2, which had been equilibrated with 0.7 M sodium acetate solution in the cold. Elution at 3° with 0.7 M sodium acetate solution removed a cloudy solution of lipovitellin, which was precipitated by dialysis, but left phovitin in the column.

Portions (10 ml) of lipovitellin in 0.7 M sodium acetate solution were transferred to dialysis bags and dialyzed against distilled water in the cold room until all salts were removed. The suspensions were quantitatively transferred to weighed 30-ml beakers and lyophilized to remove water. The dry beakers and contents were weighed, and the concentration of lipovitellin in the solution was calculated. The dry lipovitellin was extracted with three 10-ml portions of chloroform–methanol (1:1, v/v) to remove lipid, and the extracted lipid and residual protein (vitellin) were weighed to determine the per cent of lipid in the lipoprotein.

Determination of Amino Acids. Proteins were hydrolyzed with constant-boiling hydrochloric acid in an evacuated, sealed tube at 110° for 24, 48, 72, and 96 hr. Peptides were hydrolyzed in the same manner for 24 hr. The hydrolysate was transferred to a small beaker, and acid was removed by evaporation on the steam bath. Distilled water was added to the residue two times and was removed each time by evaporation on the steam bath to remove excess HCl. Amino acid content of the hydrolysates was determined with the Technicon amino acid analyzer using a procedure based on the Piez and Morris (1960) modification of the Spackman *et al.* (1958) method. Part of the analyses was made using the long column with Type A Chromobeads making one run every 24 hr, and part was done on three short columns with Type B Chromobeads making three runs every 24 hr. The ninhydrin reagent was prepared daily for more consistent results.

Separation of Lipids by Chromatography on Silicic Acid Columns. The procedure used was based on the methods described by Rhodes (1958) and by Barron and Hanahan (1958). The lipids extracted from lipovitellin by chloroform–methanol (1:1, v/v) or from lipovitellin or lipovitellin digests by ether were dissolved in hexane (Skellysolve B, obtained from the Skelly Oil Co.) and applied to a column of silicic acid (Mallinckrodt, 100 mesh powder, suitable for chromatographic analysis by the method of Ramsey and Patterson). Hydrocarbons were eluted with hexane, sterol esters with 15% benzene in hexane, triglycerides with 5% diethyl ether in hexane, sterols with 20% diethyl ether in hexane, mono- and diglycerides with diethyl ether, cephalins with chloroform–methanol (70:30, v/v), and lecithins with methanol–chloroform–water (75:20:5, v/v). All operations were carried out under nitrogen. The eluted lipid solutions were evaporated, and the lipids were dried and weighed. The identity of each fraction was verified by thin-layer chromatography.

Thin-Layer Chromatography of Lipids. Thin-layer chromatographic plates were prepared and chromatography was run according to the procedures described by Mangold (1961). Ether extracts of lipovitellin, or of the enzymatic digests of lipovitellin, and samples of standard lipids were dissolved in hexane and spotted on plates coated with silica gel G or H (E. Merck Ag., Darmstadt, Germany). Neutral lipids were separated by developing the silica gel G plate with hexane–diethyl ether–acetic acid (90:10:1) (Mangold and Malins, 1960) and phospholipids were separated by developing the silica gel H plate with chloroform–methanol–water (65:25:4) (Wagner, 1960). Lipid spots were visualized by spraying the silica gel G plates with a solution of 0.2% 2′,7′-dichlorofluorescein in 95% ethanol and the silica gel H plates with a solution of Rhodamine B (0.05 g/100 ml) in 95% ethanol and viewing under ultraviolet light.

Extraction of Lipids from Solution of Lipovitellin. Solutions of lipovitellin in 0.7 M sodium acetate were dialyzed against distilled water to remove sodium acetate, and sodium chloride and water were added to give a solution containing 10 mg of lipovitellin/ml in 1.0 M sodium chloride solution. Lipovitellin solution (10 ml) or 1.0 M sodium chloride solution (10 ml) was transferred to a 60-ml separatory funnel and then treated with either 8 M urea, 6 M guanidine-HCl, 0.05 M sodium thioglycolate, 0.0026 M sodium dodecyl sulfate, 1.0% sodium cholate, 2.0% sodium deoxycholate, or 1.0% Brij-35, Tween-80, or Triton X-100 before extraction or extracted without treatment. Each solution was extracted three times with 20-ml portions of diethyl ether. Untreated lipovitellin solutions were also extracted in the same way with chloroform–methanol (1:1), ethanol–ether (3:2), or heptane. Lipovitellin (10 ml) solution was added to 150 ml of methylal–methanol (4:1), the solution was allowed to stand for 1 hr, and the insoluble peptide was removed by centrifugation. Solvent was removed from the lipid, and the dry lipid was weighed.

Enzymatic Digestion with Phospholipases. A solution of lipovitellin containing 23 mg/ml in 1.0 M sodium chloride solution was prepared. To 10 ml of lipovitellin solution were added 1 ml of pH 7.4 boric acid–borax buffer (0.2 M borate), 1 ml of 0.02 M calcium chloride solution, and 1 ml of phospholipase C (100 mg dissolved in 7 ml of H_2O, Sigma Chemical Co., from *Clostridium welchii*, type 1). The mixture was incubated with shaking at 40° for 8 hr.

To 10 ml of the lipovitellin solution were added 1 ml

of 0.5 M acetate buffer of pH 5.6, 1.0 ml of 0.02 M calcium chloride solution, and 1 ml of solution of phospholipase D (100 mg dissolved in 7 ml of H_2O, Sigma Chemical Co., from cabbage, type II). The mixture was incubated with shaking at 40° for 8 hr.

Digestion with Proteolytic Enzymes. The solution of lipovitellin (10 mg/ml) in 0.7 M sodium acetate solution was dialyzed to remove sodium acetate, and ammonium bicarbonate was added to give a 1% solution of pH 8.5. To this were added 0.1 mg of trypsin (Worthington Biochemical Corp., two-times crystallized)/ml and a few drops of toluene, and the mixture was incubated at 37° with shaking for 24 hr. The residue from the trypsin digestion was suspended in 0.2 M pyridine–acetate buffer of pH 5.5, and 0.13 mg of ethylenediaminetetraacetic acid/ml, 0.73 mg of cysteine/ml, 0.13 mg of papain (suspension of two-times-crystallized papain, Sigma Chemical Company)/ml, and a few drops of toluene were added. The mixture was incubated with shaking at 37° for 24 hr. The residue from papain digestion was suspended in 1% ammonium bicarbonate buffer of pH 7.7, 0.01 mg of Pronase (45,000 PUK/g, obtained from Kingsley and Keith or Calbiochem)/ml, and a few drops of toluene were added, and the mixture was incubated with shaking at 37° for 48 hr.

Extraction of Lipids from Digest. Enzymatic hydrolysates were transferred to separatory funnels and extracted three times with two volumes of diethyl ether. The ether layer was transferred to a weighed beaker, and the ether and residual moisture were removed by evaporation at room temperature and then under vacuum. The dried lipid was weighed.

Separation of Peptides from Residue. The ether-extracted enzymatic hydrolysates were centrifuged for 30 min at 17,000 rpm in a refrigerated centrifuge. The peptide solution was decanted from the pellet of insoluble lipoprotein. The soluble peptides were lyophilized and weighed. The insoluble residue was suspended in buffer and then hydrolyzed with another of the proteolytic enzymes.

Results

Chloroform–methanol (1:1) extracted 24.0% of the lyophilized lipovitellin leaving 76.0% of the apoprotein vitellin. Vitellin prepared in this way contained 13.7% nitrogen and 0.9% phosphorus. Vitellin contained high levels of acidic amino acids (21.4%) and basic amino acids (16.6%) and about 13.0% of hydroxy acids; 51% of the amino acids contained polar groups The lipids were composed of 0.8% hydrocarbons, 54.2% phospholipids, 5.6 sterols and esters, and 39.4% glycerides.

Extraction of Lipid from Lipovitellin. The lipid was completely extracted from lipovitellin with chloroform–methanol (1:1, v/v), but methylal–methanol (4:1) only extracted 88%, and ether–ethanol (2:3) 53%. Ether by itself extracted 17% of the lipid but heptane did not extract any.

Treatment of the lipovitellin with either 8 M urea or 6 M guanidine hydrochloride did not increase the lipid extracted by ether, which indicates that hydrogen bonding is not primarily responsible for binding of lipids to

TABLE I: Extraction of Lipids by Ether from Lipovitellin after Treatment with Various Materials.[a]

Treatment	% of Total Lipid
None	17
8.0 M urea	6
6.0 M guanidine-HCl	4
0.05 M sodium thioglycolate	14
1.0% Brij-35	7
1.0% Tween-80	0
1.0% Triton X-100	0
0.0026 M sodium dodecyl sulfate	45
1.0% cholate	8
2.0% deoxycholate	30
1.0% cholate + 2.0% deoxycholate	30
1.0% cholate + 2.0% deoxycholate + 0.0026 M sodium dodecyl sulfate	27
Lyophilization	20

[a] Lipovitellin (100 mg) dissolved in 10 ml of 1.0 M sodium chloride solution was extracted three times with 20-ml portions of diethyl ether. Where the lipovitellin was first treated, an amount of the denaturing agent or detergent required to give the desired concentration was added to the lipovitellin solution and the blank some time before extraction.

TABLE II: Fractions[a] of Lipids in Total Lipid (chloroform–methanol extract) and in Ether Extracts from Lipovitellin and Lipovitellin Treated with 2.0% Deoxycholate (per cent of total lipids).

Lipid	Chloroform–Methanol Extract	Ether Extract of Lipovitellin	
		No Treatment	Deoxycholate
Hydrocarbons	0.8	1.3	0.0
Sterol esters	0.8	0.5	2.9
Triglycerides	34.2	70.9	39.7
Sterols	4.8	7.5	23.5
Mono- and diglycerides	5.2	2.0	4.4
Cephalins	12.5	4.8	2.9
Lecithins	41.7	13.0	26.5

[a] The lipids were fractionated by chromatography through silicic acid columns. Hydrocarbons were eluted with hexane, sterol esters with 15% benzene in hexane, triglycerides with 5% diethyl ether in hexane, sterols with 20% diethyl ether in hexane, mono- and diglycerides with diethyl ether, cephalins with chloroform–methanol (70:30, v/v), and lecithins with methanol–chloroform–water (75:20:5, v/v). The solvents were evaporated and the lipids were weighed.

TABLE III: Release of Lipids by Progressive Digestion of Lipovitellin with Proteolytic Enzymes[e] (per cent of total lipids extracted from lipovitellin by ether after enzymic treatment).

Enzyme	Lyophilized[a]	Lyophilized[b]	Lyophilized[c]	Native[d]
None	29.8	22.1	25.1	37.0
Trypsin	42.7	47.5	13.2	28.5
Papain	20.2	27.1	58.1	16.0
Pronase	7.3	3.3	3.6	18.5

[a] Freshly prepared lipovitellin (Cambridge); average of five experiments. [b] Year-old lipovitellin (Cambridge); average of four experiments. [c] Freshly prepared lipovitellin (M. S. U.); one experiment. [d] Average of four experiments. [e] Lipovitellin was extracted with ether and the ether-extracted lipovitellin was digested progressively with trypsin, papain, and Pronase. Each digest was extracted with ether, and the soluble peptides were separated from the insoluble residue by centrifugation. Ether was evaporated, the peptides were lyophilized, and the dried materials were weighed.

protein in lipovitellin (Table I). Pretreatment of the lipovitellin with 0.05 M sodium thioglycolate did not increase lipid extracted by ether. The nonionic detergents, Brij-35, Tween-80, and Triton X-100, actually decreased the lipid that was extracted by ether. Anionic detergents, as represented by 0.75% sodium dodecyl sulfate and 1% sodium deoxycholate, affected the lipovitellin so that an additional 10–30% of the total lipid was extracted by ether. The anionic detergents appeared to release all of the sterols and sterol esters from lipovitellin so that they were extracted with ether (Table II).

Digestion of lipovitellin with phospholipase C or with phospholipase D did not increase the amount of lipid extracted by ether. Either the enzymes could not reach the phospholipids because they were buried in the lipovitellin molecule or the enzymatic action did not free the products of hydrolysis from the molecule.

More lipid was extracted from dry lyophilized lipovitellin (Table III) than was extracted from solutions of lipovitellin in 1.0 M sodium chloride. More lipid was extracted from a suspension of native lipovitellin in water than from the lyophilized lipoprotein (Table III).

Proteolytic Digestion of Lipovitellin. Less lipid was released by trypsin and more lipid was released by papain from the lyophilized lipovitellin prepared in Michigan than from that prepared in England (Table III). Less lipid was released by trypsin and papain digestion from the native than from the lyophilized lipovitellin. About one-third of the lipid was extracted from a water suspension of native lipovitellin before proteolytic digestion, another third was extracted after trypsin digestion, and about one-sixth after papain digestion and the other sixth after Pronase digestion. The lipids extracted from undigested lipovitellin were mainly triglycerides (Table IV). The composition of the lipids released by trypsin, papain, and Pronase were different from each other and from those extracted before digestion.

Trypsin hydrolysis of either lyophilized or native lipovitellin hydrolyzed 76–85% of the protein to soluble peptides (Table V). Papain hydrolysis released an additional 12–18% of the protein, and Pronase released 0–4%, leaving an insoluble residue of 1–5% of the protein.

Total peptides released by each of the enzymatic digestions were analyzed for amino acids. Peptides released by papain contained more threonine, glutamic acid, alanine, isoleucine, leucine, and histidine and less serine, proline, and arginine than did those released by trypsin (Table VI). Peptides released by Pronase contained more threonine, alanine, valine, isoleucine, leucine, and phenylalanine and less aspartic acid, glutamic acid, and arginine than did those released by trypsin and more serine, proline, glycine, valine, methionine, leucine, and arginine and less aspartic acid and glutamic acid than did those released by papain.

An experiment was conducted using three successive Pronase digestions of lyophilized and native lipovitellin rather than successive digestion with trypsin, papain, and Pronase. Lipids released by trypsin or Pronase di-

TABLE IV: Fractions of Lipids in Ether Extracts of Native Lipovitellin Fractions Obtained by Progressive Digestion with Proteolytic Enzymes[a] (per cent of total lipid extracted).

Lipid	Enzyme			
	None	Trypsin	Papain	Pronase
Hydrocarbon	1.3	1.5	2.6	7.0
Sterol esters	0.5	1.7	0.4	6.1
Triglycerides	70.9	27.5	16.2	15.7
Sterols	7.5	5.0	4.4	10.3
Mono- and diglycerides	2.0	0.6	12.2	17.3
Cephalins	4.8	11.4	18.4	14.4
Lecithins	13.0	52.3	45.8	29.2

[a] See footnote *a* of Table II.

TABLE V: Release of Peptides by Progressive Digestion of Lipovitellin with Proteolytic Enzymes[c] (per cent of total protein released as soluble peptides).

Enzyme	Lyophilized[a]	Lyophilized[b]	Native[b]
Trypsin	75.8	85.4	79.4
Papain	17.6	13.5	11.9
Pronase	3.3	0	4.0
Residue	3.3	1.2	4.7

[a] Year-old lipovitellin (Cambridge). [b] Freshly prepared lipovitellin (M.S.U.). [c] See footnote *e* of Table III.

TABLE VI: Amino Acid Content of Total Protein and of Peptide Fractions Released from Native Lipovitellin by Progressive Proteolytic Digestion[a] (grams of amino acid in 100 g of protein N × 6.25).

Amino Acid	Total Protein	Trypsin Released	Papain Released	Pronase Released	Residue
Aspartic acid	7.7	8.0	8.1	3.8	7.0
Threonine	4.0	3.8	5.4	5.2	5.7
Serine	5.7	6.6	5.4	7.0	5.7
Glutamic acid	13.7	11.4	12.4	10.2	12.3
Proline	4.6	5.0	0.2	4.5	5.0
Glycine	3.0	2.8	2.3	3.2	2.2
Alanine	5.2	4.6	6.4	6.2	6.3
Valine	7.0	5.8	6.5	8.0	8.0
Methionine	1.9	2.0	1.8	3.0	6.3
Isoleucine	6.1	4.4	6.0	6.8	7.8
Leucine	8.5	7.6	10.0	11.2	12.7
Tyrosine	3.3	3.9	4.0	4.2	3.5
Phenylalanine	3.8	3.4	4.0	4.8	4.0
Lysine	6.5	6.6	6.4	6.2	5.8
Histidine	2.3	2.2	3.0	3.0	2.7
Arginine	7.8	8.3	4.6	6.0	5.3

[a] Amino acids were determined on the acid-hydrolyzed proteins with a Technicon amino analyzer. Vitellin was hydrolyzed in evacuated tubes with 20% HCl for 24, 48, 72, and 96 hr at 110°, and peptides were similarly hydrolyzed for 24 hr.

gestion of lyophilized lipovitellin were similar in amount, but papain digestion of the residue released more lipid than did a second Pronase digestion (Tables III and VII). For some reason less lipid was extracted by ether from the native lipovitellin used for Pronase digestion than from that used for trypsin digestion, but the first Pronase digestion released more lipid than did trypsin digestion; 13.9% of the lipid remained after the first Pronase digestion compared with 34.5% after trypsin digestion.

Similar amounts of peptide were released from lyophilized lipovitellin by three successive Pronase digestions as by digestion with trypsin, papain, and Pronase (Tables V and VII). Trypsin released more peptide from native lipovitellin than did the first Pronase digestion, and the residues from the second and third Pronase digestions were larger than those from papain and from Pronase digestion of the papain residue.

Progressive digestion of unextracted native lipovitellin with trypsin, papain, and Pronase was carried out to study the affinities of the undigested residues for lipid. A suspension of lipovitellin in a 1% ammonium bicarbonate solution of pH 8.5 was digested by trypsin, and the soluble peptides were separated from the insoluble residue by centrifugation. The residue was suspended in 0.2 M pyridine–acetate buffer of pH 5.5 and digested with papain, and the peptides and residue were separated. The residue left after papain digestion was suspended in 1% ammonium bicarbonate solution of pH 7.7 and digested with Pronase. Each peptide fraction was extracted with ether to separate the released peptides and lipids. Comparison of data in Table VII with that in Tables III and V show that some of the lipids that could be normally extracted with ether remained with the residues after trypsin and papain digestions of lipovitellin, when the digests were not extracted with ether, and that the lipids partially protected the lipoprotein or residue from proteolytic digestion. The residue from trypsin digestion of extracted lipovitellin contained 65% protein and 35% lipid compared

TABLE VII: Release of Lipids and Peptides by Progressive Digestion of Lipovitellin with Pronase[b] (per cent of total lipid or protein).

	Lipids		Peptides	
Digestion	Lyophilized[a]	Native	Lyophilized[a]	Native
None	26.4	12.9		
First	53.3	73.3	72.1	65.6
Second	12.6	7.1	20.5	19.9
Third	7.7	6.8	2.6	7.0
Residue			4.9	7.5

[a] Year-old lipovitellin (Cambridge). [b] Lipovitellin was digested with Pronase, the digest was extracted with ether and then centrifuged to separate the soluble peptides and the residue, the residue was again digested with Pronase, and the procedure was repeated for a total of three Pronase digestions.

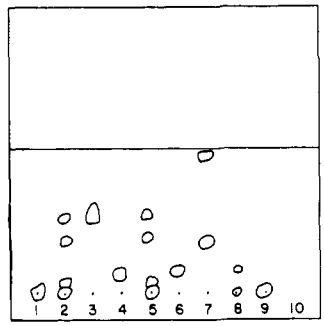

FIGURE 1: Thin-layer adsorption chromatography of lipid classes on silica gel G. Solvent: hexane–diethyl ether–acetic acid (90:10:1). Indicator: dichlorofluorescein. (1) Monolein, (2) chloroform–methanol extract of lipovitellin residue, (3) triolein, (4) cholesterol, (5) chloroform–methanol extract of LDL residue, (6) diolein, (7) cholesterol stearate, (8) chloroform–methanol extract of blank residue, and (9) lecithin.

with 57% protein and 43% lipid in the unextracted residue. The extracted residue after papain digestion contained 60% protein and 40% lipid compared with 47% protein and 53% lipid in the unextracted residue.

The ether-extracted residue remaining after trypsin, papain, and Pronase digestion of native lipovitellin was not all protein as has been assumed for the previous calculations in the present study. Kjeldahl analyses showed it to contain 56.6% of protein (N × 6.25). Chloroform–methanol extracted 32.8%, which is reported as lipid, and there was 1.0% carbohydrate (determined by procedure of Synge and Wood, 1958) leaving 9.6% of unknown material. The residue protein contained less aspartic acid, serine, lysine, and arginine and more threonine, alanine, valine, methionine, isoleucine, and leucine than did the peptides released by trypsin. The residue contained 19.3% acidic, 14.9% hydroxy, 13.8% basic, and 52.3% neutral amino acids compared with 19.4% acidic, 14.3% hydroxy, 17.1% basic, and 35.6% neutral amino acids in the peptides released by trypsin (Table VI). The residue thus contained a higher proportion of neutral and a lower proportion of basic amino acids. The lipids of the residue contained cholesterol esters, triglycerides (Figure 1), cephalins, lecithins (Figure 2), and possibly mono- and diglycerides, which migrated by thin-layer chromatography as did cholesterol and phosphatides in thin-layer plates developed with hexane–diethyl ether–acetic acid (Figure 1) or triglyceride on plates developed with chloroform–methanol–water (Figure 2).

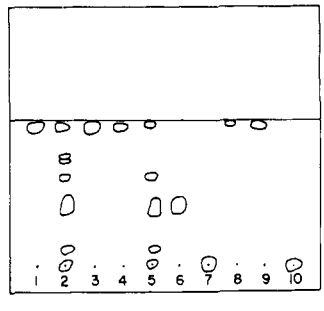

FIGURE 2: Thin-layer adsorption chromatography of lipid classes on silica gel H. Solvent: chloroform–methanol–water (65:25:4). Indicator: Rhodamine B. (1) Dipalmitin, (2) chloroform–methanol extract of lipovitellin residue, (3) triolein, (4) cholesterol, (5) chloroform–methanol extract of LDL residue, (6) lecithin, (7) cephalin, (8) chloroform–methanol extract of blank residue, (9) cholesterol stearate, and (10) phosphatidylethanolamine.

Discussion

It appears from the data herein presented that a variable part of the lipid of lipovitellin is bound in some way on the surface of the molecule so that it can be extracted readily by ether but not by heptane. Most of the lipid, however, appears to be held within the protein network either as separate molecules, as layers of molecules, or as groups of molecules in pockets where they are shielded from the action of ether. Most of the data obtained can be explained by some model of the above type.

According to Cook and Wallace (1965) the monomer of lipovitellin has a molecular weight of 227,000 and a lipid content of 20% from which the vitellin (apoprotein) portion would have a molecular weight of 181,600. Progressive digestion of native lipovitellin with trypsin, papain, and Pronase left a residue equivalent to 4.7% of the vitellin. Since this residue was 56.6% protein, the residual protein was equivalent to 2.7% of the vitellin or an insoluble peptide with a molecular weight of 4900. Calculations using data of Table VI for the amino acid content of the residue protein indicate the peptide to have the following empirical formula $Asp_3Thr_3Ser_3Glu_5Pro_3Gly_2Ala_4Val_4Met_2Ile_3Leu_6TyrPhe-Lys_2HisArg_2$. Similarly the lipid portion can be shown to have a molecular weight of 2630 probably made up of one molecule each of triglyceride, sterol, cephalin, and lecithin. The lipid must be held to the peptide by quite strong bonds or buried within the folds of the peptide because it is not extracted from the peptide by ether. Additional information on the binding of this lipid to the peptide portion must await further studies of the residue.

The composition of the residue from the trypsin and papain digestion of lipovitellin can similarly be calculated to be composed of peptide with a molecular weight of 12,340 and lipid of mol wt 12,700. The peptide was calculated as $Asp_6Thr_7Ser_9Glu_{11}Pro_7Gly_6Ala_{11}Val_{10}Met_4Ile_8-Leu_{13}Phe_3Lys_6His_3Arg_5$ and the lipid as hydrocarbon, sterol ester, $triglyceride_3$, $sterol_4$, mono- and $diglyceride_4$, $cephalin_3$, $lecithin_4$. Most of the lipid is held within the peptide chain so that it cannot be extracted by ether until part of the peptide is removed by Pronase digestion. Papain digestion of the residue from trypsin digestion of lipovitellin released peptides of total molecular weight of 22,000 and lipid of molecular weight of 8780. Trypsin digestion of the ether-extracted lipovitellin released by far the most of the protein as soluble peptide (147,260 total molecular weight) but a smaller proportion of lipid (15,560 molecular weight).

The lipid bound to the outside of the native lipovitellin molecule (Tables III and IV) and readily extracted by ether, consisted of 17 molecules of triglyceride, 4

molecules of sterol, 4 molecules of lecithin, and 1 molecule each of cephalin and mono or diglyceride.

The amount of lipid that is bound to lipovitellin so that it is extracted by ether appears to be variable. For example, 17% of the total lipid was extracted from a solution of native lipovitellin in 1.0 M sodium chloride solution (Table I), while 22–30% was extracted from dry lyophilized lipovitellin and 37 (Table III) or 13% (Table VII) from suspensions of native lipovitellin in water. The differences are probably caused by the differences in the amount of this loosely bound lipid that remained with the lipovitellin during the preparation procedure. Each one of these determinations was made using a different lipovitellin preparation, and a different preparation was used in each of the four experiments using native lipovitellin (Table III). The freshly prepared Cambridge lipovitellin contained more loosely bound lipid than it did after standing in a lyophilized condition 1 year. The reason for this is not known.

Urea (8.0 M), guanidine-HCl (6.0 M), or (1.0%) sodium cholate treatment of a solution of native lipovitellin in 1.0 M sodium chloride appeared to stabilize the binding of some lipids to the protein in as much as they decreased the amount of lipid extracted by ether. The nonionic detergents in most cases completely prevented the extraction of lipid from lipovitellin and appeared to stabilize the binding (Table I). Ether, which extracted this surface lipid, is more polar than heptane, which did not extract it.

Progressive proteolytic digestion of unextracted native lipovitellin sheds further light on the lipid–protein relationships. In the first place the lipid on the outside of the lipovitellin molecule partially protects it from proteolytic digestion, because trypsin digestion of the unextracted lipovitellin released 60.7% of the protein as soluble peptide and digestion of the extracted lipovitellin released 79.4% (Tables V and VIII). Trypsin digestion followed by papain solubilized 73.4% of the protein of unextracted lipovitellin while it solubilized 93.4% of the extracted lipovitellin. Only 4.6% of the lipid was released from the unextracted lipovitellin by trypsin digestion and the rest remained with the insoluble lipoprotein. Ether extracted 65.5% of the lipids of the trypsin-digested lipovitellin. There appears to be a strong affinity between lipid and peptide even after more than half of the protein was removed by trypsin digestion. In fact after the removal of 73.4% of the protein by digestion of the unextracted lipovitellin with trypsin followed by papain, 94.1% of the lipid was still associated with the insoluble peptide. There appears to be a strong affinity of protein for lipid all through the lipovitellin molecule.

Hydrophobic bonding appears to be involved either in the binding of part of the lipids to protein or in holding the protein molecule in shape so that the lipids are enclosed. An additional 54% of the lipid was extracted by ether when lipovitellin was treated with sodium dodecyl sulfate, probably by opening up part of the peptide chain so that ether could reach pockets of lipids.

Burley (1963) observed that lipovitellins absorbed chloroform from chloroform aqueous buffer solutions, that the chloroform absorption process produced a heavier fraction that was not homogeneous and probably consisted of aggregates, and that 13–22% of the dry weights of lipovitellins may be absorbed. Absorption of chloroform by lipovitellin appears to open up the molecule in some way because while phospholipase C did not act on native lipovitellin, after absorption of chloroform by lipovitellin a large proportion of the phospholipids were hydrolyzed (Burley and Kushner, 1963).

TABLE VIII: Release of Peptides and Lipids by Progressive Proteolytic Digestion of Unextracted Native Lipovitellin[a] (per cent of total of each).

Enzyme	Peptide	Lipid
Trypsin	60.7	4.6
Papain	12.7	1.4
Pronase	12.2	86.1
Residue	14.4	8.0

[a] Unextracted lipovitellin was digested with trypsin; the soluble peptides were separated from the insoluble residue by centrifugation, and the residue was digested with papain. The residue from the papain digestion was digested with Pronase. The peptides were extracted with ether as was the residue from the Pronase digestion.

Bernardi and Cook (1960a) observed that lipovitellin could be resolved electrophoretically into two components, which they termed α- and β-lipovitellin. α- and β-lipovitellins were separated from each other by chromatography on hydroxylapatite columns and were shown to have the same lipid content, nitrogen content, amino acid content, and molecular weight (Bernardi and Cook, 1960b). They differed in protein phosphorus content (Bernardi and Cook, 1960b; Burley and Cook, 1961), urea concentration required for dissociation (Bernardi and Cook, 1960c), pH change required for dissociation (Burley and Cook, 1962; Kratohvil *et al.*, 1962), content of histidine (Cook *et al.*, 1962), and amount of chloroform absorbed from chloroform-saturated buffer solutions (Burley, 1963). Total lipovitellins were used in the present studies because the α- and β-lipovitellins were so nearly similar that it was felt that there would not be enough differences to warrant the extra work required to separate the two constituents in these early studies.

Human blood plasma α-lipoprotein, the high-density lipoprotein, was subjected to enzymatic hydrolysis by Ashworth and Green (1963). Phospholipase D digestion of α-lipoprotein released 88% of the choline and made 79% of the sterol and 51% of the phospholipid extractable with ether, but phospholipase D had no effect on lipovitellin. Trypsin digestion appeared to hydrolyze similar amounts of protein from α-lipoprotein and lipovitellin, but more lipid appeared to be exposed to ether extraction in α-lipoprotein than in lipovitellin. Thus, there are differences between the structures of lipovitellin and of human blood α-lipoprotein.

In conclusion lipovitellin appears to consist of a protein chain folded in a globular configuration with lipid, equal to one-fourth of the weight of the protein held in pockets throughout the molecule. A variable amount of lipid is held on the surface of the molecule. The protein appears to be held in shape primarily by hydrophobic bonds, and when some of these are broken by treatment with sodium dodecyl sulfate or sodium deoxycholate, part of the lipid held within the molecule is exposed so that it can be extracted with ether. Proteolytic digestion hydrolyzes part of the protein to soluble peptides and leaves a residue with lipid on the surface. Removal of the lipid with ether then exposes peptide groups susceptible to further proteolytic hydrolysis. The amount of lipid held on the outer surface of the lipovitellin molecule appears to depend upon the method of preparation, solvent composition, and other unknown variables.

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