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Lipovitellin

4. CHEMICAL COMPOSITION OF THE LIPID AND OF THE FRACTIONS REMOVED BY VARIOUS TREATMENTS

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The marked tendency for definite proportions of the total lipid of lipovitellin to be removed by different physical and chemical treatments (Lea & Hawke, 1952) suggested an investigation of the chemical nature of the lipid fractions so removed. Very few investigations of this nature appear to have been carried out, and these have usually been based on variations of the extraction procedure of McFarlane (1942). Delsal (1949) stated that 'delipidation' of blood serum by anhydrous ether at -70° extracted a considerable proportion of the cholesterol, apparently without freeing any of the phospholipin from its association with protein. Oncley, Gurd & Melin (1950) extracted human serum β -lipoprotein (in which phospholipin constituted 38% of the total lipid—itsself 77% of the lipoprotein), with ether at -50° rising to 0° and removed in the ethereal layer about three-quarters of the total lipid containing a smaller proportion (about 28%) of phospholipin. The remaining lipid which was removed practically quantitatively by subsequent extraction of the aqueous phase and emulsion with ethanol-ether contained 90% of phospholipin. The thromboplastic lipoprotein of lung has been shown to lose about one-third of its lipid (rich in acetal phospholipin) by similar treatment (Chargaff & Bendich, 1944). Since the completion of the present work Cohn, Surgenor, Schmid, Batchelor & Isliker (1952) have briefly

reported quantitative extraction of cholesterol from the α -lipoprotein of human serum by perfusion with ether or petroleum ether, apparently without extracting any of the lipid phosphorus.

The lipids of hens' eggs form 30–35% of the weight of the yolk (61–71% of the dry weight) and rather less than one-third of these are phospholipins. Phosphatidylcholine is usually considered to be a major constituent of egg phospholipin, but estimates of the content of the 'kephalin' fraction vary widely (Wittcoff, 1951).

Chargaff (1942) found that the composition of the phospholipin bound in lipovitellin approximated to that of the free phospholipins of the yolk.

EXPERIMENTAL

Materials

All the lipid fractions were prepared from hens' eggs collected from the same flock over a short period. Fractions A to H were from the same batch of mixed egg yolk and fractions B1 and C to H from the same preparation of lipovitellin (total lipid content = 29.8%). Lipid fraction J was prepared from a lipovitellin containing 31.5% lipid (B2) and fraction K from a lipovitellin containing 30.2% lipid (B3). Since variations between the compositions of the lipids from the three specimens of lipovitellin were within the limits of error of the determinations the values have been averaged (Table 1). Methods used for preparation of the

individual fractions were based on a previous investigation of the properties of lipovitellin (Lea & Hawke, 1952) as follows:

A. *Total lipids of egg yolk.* The yolk was freeze-dried and extracted in a Soxhlet apparatus with hot ethanol for 4 hr.

B. *Total lipid of lipovitellin.* Lipovitellin prepared as previously described (Lea & Hawke, 1952) was freeze-dried and extracted with hot ethanol as above.

C. *Lipid extractable by ether from lipovitellin freeze-dried at pH 6.8.* Freeze-dried lipovitellin was shaken with ether (200 ml./g.) for 30 min. at 0° and the extracted lipid recovered by evaporating the filtrate *in vacuo* at room temperature (=23% of B).

D. *Lipid extractable by ether from lipovitellin freeze-dried at pH 5.2.* As fraction C (=21% of B).

E. *Lipid extractable by ethanol-ether at -7° from lipovitellin freeze-dried at pH 6.8.* Lipid removed by four 30 min. extractions with ethanol followed by two 30 min. extractions with ether was recovered as for fraction C (=83% of B).

G. *Lipid extractable by ether from lipovitellin freeze-dried at pH 6.8 and stored 5 days at 37° and 85% relative humidity (R.H.).* As fraction C (=32% of B).

H. *Lipid extractable by ether from lipovitellin freeze-dried at pH 6.8 and stored 5 days at 37° and 92% R.H.* As fraction C (=40% of B).

J. *Lipid extractable from an aqueous suspension of lipovitellin in the presence of 0.05 M-NaCl.* A 10% (w/v) aqueous suspension of lipovitellin at pH 6.8, 0.05 M in respect of NaCl, was shaken with ether (200 ml./g. lipoprotein) for 30 min. at 0° and the extracted lipid recovered as above (=32% of B).

K. *Lipid extractable from lipovitellin suspension by freezing in the presence of ether.* A 10% (w/v) aqueous suspension of lipovitellin at pH 6.8 was emulsified with ether (2 ml./3 g. suspension) and frozen at -70° for 15 min. The temperature was then allowed to rise to 0° and the free lipid removed by extracting with ether for 30 min. at 0° (=9% of B).

The lipid fractions were all dried for analysis by the repeated removal of small volumes of ethanol *in vacuo*, dissolved in dry ether, distributed in glass ampoules from which the solvent was subsequently removed, exhausted for several hours *in vacuo*, sealed and held at -30° until required.

Analytical methods

Separation of phospholipin from non-phospholipin. The lipid samples were dissolved in the minimum volume of petroleum ether and the phospholipins precipitated quantitatively by the addition of acetone and ethanol saturated with $MgCl_2$ (Fairbairn, 1945), followed by standing overnight at 0°.

Total phospholipin and non-phospholipin. Total N was determined in the precipitate by the micro-Kjeldahl procedure and phosphorus by the colorimetric method of Allen (1940) after digestion with perchloric acid.

The average molecular weight of purified egg lecithin is usually about 800 (Faure & Legault-Demare, 1950), and a weighted mean between phosphatidylcholine and phosphatidylethanolamine, which would be 60 units lower, gives 780 as an approximate average molecular weight of egg phospholipin. For the purposes of the present work phospholipin content has been calculated from lipid P by use of the factor 25 (780/31 approx.). Plasmalogens (approximate molecular weight 450) which would introduce

serious errors into this form of calculation are believed to be virtually absent from egg (Feulgen & Bersin, 1939).

Non-phospholipin was determined gravimetrically in the acetone-soluble fraction after evaporation to dryness and re-solution in ether.

Choline. Choline was determined by the method of Entenman, Taurog & Chaikoff (1944), and converted to phosphatidylcholine by use of the factor 6.4 (there is little or no sphingomyelin in egg).

Amino nitrogen. Folch, Schneider & Van Slyke (1940) reported that unhydrolysed phospholipin gives abnormally high amino-N values by the manometric Van Slyke procedure owing to the liberation of nitrogen from nitrous acid by the unsaturated fatty acids present. Kline, Glegg & Sonada (1951), on the other hand, were able to obtain similar values from unhydrolysed and hydrolysed phospholipins by suitable choice of reaction time. In our experience, values obtained on unhydrolysed material were as much as 25% higher than those obtained after hydrolysis and all lipid fractions were therefore subjected to methanolysis by refluxing with 6N-HCl in dry methanol (10 ml./100 mg. phospholipin) for 3 hr. (Artom, 1945). Following repeated evaporation *in vacuo* at 35-40° the residue was refluxed with water (10 ml./100 mg. phospholipin) to hydrolyse any serine ester formed during methanolysis and the cooled mixture extracted several times with ether to remove the fatty esters. Free amino N was determined in the aqueous phase after concentration. Egg-yolk phospholipins contain no serine (Chargaff, Ziff & Rittenberg, 1942), and it has been considered sufficiently accurate for the present purpose to convert amino N to phosphatidylethanolamine by use of the factor 55.4.

Cholesterol. Free and total cholesterol were determined by the digitonin precipitation method of Popják (1943).

Fatty acids. The methyl esters of the constituent fatty acids were recovered from several of the lipid fractions by extraction of the hydrolysates with ether and examined by determination of the iodine value by the Wijs method and of the unsaturated fatty acids by the spectrophotometric method after isomerization with alkali.

Chromatography. Paper chromatography of the water-soluble nitrogenous constituents of the phospholipin fractions, hydrolysed as described above, was carried out by the method of Levine & Chargaff (1951). After development for 18 hr. at 20° with *n*-butanol-diethylene glycol-water (4:1:1 by vol.) the papers were sprayed with (a) 0.1% (w/v) ninhydrin solution in *n*-butanol, followed by drying at 100° for 10 min. to indicate the position of bases containing amino groups or (b) 1% (w/v) phosphomolybdic acid in ethanol-chloroform (1:1 by vol.) followed by washing with running water for 15-20 min., drying at 20° and reduction with a 1% (w/v) solution of $SnCl_2$ in 3N-HCl to detect choline.

RESULTS AND DISCUSSION

For ease of comparison and because of inaccuracies inherent in the use of factors the percentage compositions of all lipid fractions have been expressed to the nearest unit.

Whilst the total lipids of egg yolk consisted of 38% phospholipin and 62% other lipids, the proportion of phospholipin in the lipid constituent of lipovitellin was very much greater (85-90%), with

Table 1. *Composition of the total lipids of egg yolk and of lipovitellin*

(Figures in brackets refer to percentage composition of the phospholipin fractions.)

		Composition (%)				
		Atomic N/P ratio	Phosphatidyl- choline*	Phosphatidyl- ethanolamine†	Total phospholipin‡	Non- phospholipin§
A	Total egg-yolk lipid	1.08	32 (84 %)	6 (16 %)	38	62
B1	Total lipid of lipovitellin (preparation 26 + 27)	1.01	75	12	87	14¶
B2	Total lipid of lipovitellin (preparation 28)	0.99	74	8	85	15
B3	Total lipid of lipovitellin (preparation 29)	1.00	77	13**	90	14
B	Total lipid of lipovitellin (average)	1.00	75 (87 %)	11 (13 %)	87	14

* Choline $\times 6.4$.
† Amino N $\times 55.4$.
‡ P $\times 25$.
§ Gravimetric.
|| Includes 4.5 cholesterol.
¶ Includes 2.4 cholesterol (1.6 esterified, 0.8 free).
** By difference (total phospholipin-phosphatidylcholine).

Table 2. *Composition of lipid fractions removed from lipovitellin by various treatments*

(Figures in brackets refer to percentage composition of the phospholipin fractions.)

		Composition of lipid fraction (%)				
Fraction (see text)	% of total lipid extracted	Atomic N/P ratio	Phosphatidyl- choline*	Phosphatidyl- ethanolamine†	Total phospholipin‡	Non- phospholipin§
B	Total lipovitellin lipids	100	75 (87 %)	11 (13 %)	87	14
C		23	31 (69 %)	14 (31 %)	50	49
D		21	29 (58 %)	21 (42 %)	50	49
E		83	67 (82 %)	15 (18 %)	84	16
G		32	58 (83 %)	12 (17 %)	70	33
H		40	53 (73 %)	20 (27 %)	73	30
J		32	53 (80 %)	13 (20 %)	69	31
K		9	58 (82 %)	13 (18 %)	71	31

* Choline × 6.4.
† Amino N × 55.4.
‡ P × 25.
§ Gravimetric.
|| By difference (total phospholipin-phosphatidylcholine).

Table 3. *Absolute quantities of lipids removed by the various treatments expressed as percentage of total lipid of lipovitellin*

		Amount of each constituent			
Fraction removed	% of total lipid	Phosphatidyl- choline*	Phosphatidyl- ethanolamine†	Total phospholipin‡	Non- phospholipin§
B	Total lipid of lipovitellin	75	11	87	14
C		23	7	12	11
D		21	6	11	10
E		83	56	70	13
G		32	19	22	11
H		40	21	29	12
J		32	17	22	10
K		9	5	6	3

* Choline × 6.4.
† Amino N × 55.4.
‡ P × 25.
§ Gravimetric.

other lipids relatively minor constituents. The ratio of phosphatidylcholine to phosphatidylethanolamine, however, was only very slightly higher in the lipovitellin, indicating an almost non-selective combination of the two classes of phospholipin with the protein (Table 1).

The 14% non-phospholipin present in the lipoprotein included only 2.4% cholesterol (0.8% free, 1.6% esterified) with the remainder probably consisting largely of neutral fat (triglycerides). Lipovitellin is thus very different from the lipoproteins of human serum in which cholesterol and its esters are major constituents.

The atomic N/P ratio of all the lipid fractions (with the exception only of the total egg-yolk lipids *A* which undoubtedly contained a certain amount of non-phospholipin nitrogen) approximated closely to 1.00 (Table 2), consistent with the presence only of monoaminophospholipins.

Table 2 shows that all the lipid fractions removed from lipovitellin by the various treatments investigated contained a considerably higher proportion of non-phospholipin than did the original total lipid of lipovitellin, removal of one-fifth to one-third of the total lipid sufficing to strip off three-quarters of the non-phospholipin, while larger fractions removed practically all of the non-phospholipin (Table 3). This behaviour is not inconsistent with the conception of phospholipins being bound to the protein by both ionic forces and van der Waals interaction with the non-polar side chains of the amino-acids, and of non-polar lipids being held by van der Waals forces to the fatty acid chains of the phospholipins (cf. Tayeau, 1944).

Of the individual phospholipins, phosphatidylethanolamine was rather less firmly held than phosphatidylcholine and all the lipid fractions removed contained relatively more of this substance than did the original total lipid (Table 2). When as much as 83% of the total lipid had been stripped off (fraction *E*) the small amount of lipid remaining bound to the protein was practically pure phosphatidylcholine (Table 3).

The union between lipid and protein in lipovitellin seems to be considerably stronger than in the blood-plasma lipoproteins. Freezing in the presence of ether, for example, removed only 9% of the lipid of lipovitellin, as compared with about 75% in the case of the β -lipoprotein of human serum, and most of the treatments employed removed considerably less than half of the total lipid. Even several extractions of the dry lipoprotein with ethanol and ether at -7° (treatment *E*, Table 3) left 17% of the original lipid (all lecithin) still attached to the protein, whereas alcohol and ether at -70° rising to 0° removed all but 1% of the lipid of human serum β -lipoprotein (Oncley *et al.* 1950).

The component fatty acids of the phospholipins

in all three fractions examined were considerably more unsaturated than those of the corresponding glycerides, the average iodine values being approximately 20 units higher (Table 4). Spectrophotometric analysis of the fatty esters showed that this

Table 4. Iodine values of the fatty-acid methyl esters from egg-yolk lipid fractions

	A Total egg-yolk lipid	B Total lipid of lipovitellin	C Lipid removed by ether extraction of freeze-dried lipovitellin
Glyceride	87.4	83.3	85.7
Phospholipin	105.8	101.3	111.6

difference was due essentially to the presence of several times as much polyunsaturated acids in the phospholipins as in the glycerides, in agreement with the observations of Riemenschneider, Ellis & Titus (1938) and of Shorland (1951). There is some doubt as to whether the polyunsaturated acids of egg lipids are arachidonic (Levene & Rolf, 1922), clupanodonic (Riemenschneider *et al.* 1938) or both (Shorland, 1951), and a sufficiently complete analysis to check the point has not been carried out in the present work. No evidence was found, however, of selective concentration of any one class of fatty acid in the lipid attached to the protein or in the fraction removed by ether extraction of the dried lipoprotein.

In the chromatographic investigation of the phospholipin hydrolysates, ethanolamine and choline were identified in all the fractions examined, i.e. in *A*, *B*, *C*, *G* and *J*. In addition, all five fractions contained traces of unidentified ninhydrin-reacting materials which may have been other bases present in very small quantity or artifacts produced during hydrolysis. The chromatographic evidence, therefore, confirmed the absence of sharp fractionation shown by the quantitative data (Table 2).

Both the recent announcement by Cohn *et al.* (1952) that cholesterol can be removed from human serum α -lipoprotein by ether and our own observations on the instability of isolated lipovitellin to ether at salt concentrations which are probably encountered during the isolation procedure (Lea & Hawke, 1952) serve to demonstrate the extremely labile nature of lipoproteins, and to stress the danger that products obtained by isolation may differ from those present in the tissues. The lipid analyses in the present work show that lipovitellin, as isolated, contains a much smaller proportion of the readily removable neutral lipids (triglyceride plus cholesterol) than does the total yolk lipid. It seems

probable, therefore, that if milder fractionation procedures could be devised a lipovitellin containing much more total lipid and a greater proportion of neutral lipids might be obtained.

SUMMARY

1. The lipid of lipovitellin contains a much higher proportion of phospholipin than does total egg-yolk lipid, but their respective phospholipins have similar proportions of phosphatidylcholine, phosphatidylethanolamine and unsaturated fatty acids.

2. The fatty acids of the phospholipins of total yolk lipid and of lipovitellin are considerably more unsaturated than are those of the corresponding glycerides.

3. Lipovitellin contains much less cholesterol than the lipoproteins of human serum and appears to be considerably more stable.

4. Analysis of fractions of the lipid which became extractable by solvents after various treatments involving freezing or drying showed that non-phospholipin was removed most readily, followed by phosphatidylethanolamine, but there was no clear-cut separation of the constituents.

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Etherification of Noradrenaline in Acidified 95 % Ethanol. (±)-2-Ethoxy-2-(3':4'-dihydroxyphenyl) ethylamine hydrochloride

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In the course of pharmacological and chemical studies on noradrenaline in these laboratories, it was observed that (–)-noradrenaline hydrogen tartrate on standing in 95 % ethanolic hydrochloric acid (1 ml. of concentrated hydrochloric acid plus 99 ml. of 95 % ethanol, hereinafter referred to as 'acid alcohol') showed evidence of forming a new compound. This evidence consisted of appearance of a second, faster-travelling spot in an ascending paper chromatogram using *n*-butanol saturated with 0.5N-hydrochloric acid as solvent and ferricyanide (James, 1948) as indicator. Since the new derivative had been formed in an acid medium it was felt that

the compound arose by reaction of the side-chain hydroxyl group. This type of reactivity was demonstrated by Tullar (1948) who has found that (±)-noradrenaline is almost quantitatively converted to its methyl ether by evaporating *in vacuo* a solution of the hydrochloride in methanol. Accordingly, it seemed reasonable to expect some ether formation on merely allowing noradrenaline to stand in acidified 95 % ethanol as well as on concentrating such solutions. It therefore was desired to synthesize the indicated ethyl ether and to demonstrate its formation under the conditions given.