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Lipovitellin

2. THE INFLUENCE OF WATER ON THE STABILITY OF LIPOVITELLIN
AND THE EFFECTS OF FREEZING AND OF DRYING

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The lipid-protein complexes (lipoproteins) of tissues are usually said to be decomposed by freezing or drying; in fact, modern theories of their structure generally include water as an integral part of the molecule (e.g. Dervichian, 1949). Little or no quantitative information on the subject has, however, been published and, in view of the increasing tendency towards preserving sensitive biological materials and foods in the frozen or dried state, an investigation of the effects on a lipoprotein of freezing and of drying has been carried out. Lipovitellin from egg yolk was chosen as experimental material for reasons already given (Lea & Hawke, 1951).

Available information on the stability of lipovitellin is conflicting. Chargaff (1942) stated that a suspension of the lipoprotein lost some of its dispersibility in sodium chloride solution on freeze-drying. Moran (1925) observed that a solution of 'lecithovitellin' in the minimum concentration of sodium chloride deposited solid on freezing and thawing. Fevold & Lausten (1946) stated that 'lipovitellin', a lipoprotein fraction from egg yolk containing 36–41% lipid, was not, by freeze-drying, rendered insoluble in sodium chloride solution saturated with ether, but that its dispersibility disappeared on standing. No information was given as to whether or not any free lipid was split off from the protein during freezing, drying or storage, and in none of these cases was the moisture content of the dried material or the conditions of freezing and thawing defined, nor were any quantitative data

recorded on the effects observed. It is, however, of considerable practical interest that commercial spray-dried egg, which has a greatly inferior aerating power to fresh egg, yields up much more of its fat on extraction with cold light petroleum than does fresh egg (Brooks & Hawthorne, 1944).

The present paper deals with some of the properties of lipovitellin in relation to the effects of freezing and drying and of storage in the frozen and dried states.

METHODS

Preparation of lipovitellin. Lipovitellin was prepared as previously described (Lea & Hawke, 1951) except that before the final dialysis any material which was not dispersible in 10% (w/v) NaCl saturated with ether was separated and discarded. It was found that if the final dialysis through cellulose at 0–2° was continued for longer than 24 hr. it became impossible, with the means available, to centrifuge down the suspended lipoprotein. The lipoprotein suspension was therefore centrifuged after dialysis for 24 hr. and the concentrated product subjected to continued dialysis with agitation from narrow cellulose sacks for a further 48 hr. at 0°.

In some preparations, 50% (w/v) sucrose was dissolved in the aqueous phase to protect the lipoprotein from denaturation by ether (Bate-Smith, 1935). This precaution could not be taken, however, in the first ether extraction of the yolk because of increased difficulty with emulsification, and was omitted from the final reprecipitation to avoid the lengthy dialysis necessary to remove the last traces of sugar from the thick lipoprotein suspension. Under these conditions, the use of sucrose resulted in no improvement in the yield or solubility of the preparation.

The lipid contents of lipovitellin preparations 18–29 used in this work were 27.5, 38.2, 38.2, 32.4, 33.2, 27.5, 25.7, 28.4, 32.1, 31.5 and 30.2% respectively.

Determination of solubility. To 2 ml. lipoprotein suspension containing approximately 200 mg. lipoprotein were added 5 ml. NaCl of the strength necessary to give the required final concentration, 0.8 ml. ether and sufficient dilute NaOH or HCl and water to produce the desired pH and a final volume of 10.6 ml. The solutions were mixed by sudden shaking, slowly tumbled for 16 hr., centrifuged, and the N content of the supernatant fluid determined by the micro-Kjeldahl procedure. For most of the routine estimations of solubility 15% (w/v) NaCl and pH 6.0 were used. All operations were carried out at 0°. Although the solubilities of fresh preparations were usually in the range 93–97%, suggesting the possibility of incomplete equilibration or of denaturation during the determination, shorter or longer times of equilibration failed to give higher results than those obtained at 8 or 16 hr.

ferred to a refrigerated freeze-drying cabinet in a room at 0°, and the apparatus rapidly evacuated. Brine at –25 to –30° circulating through the cooling column of the drier carried away the heat of hydration of the P_2O_5 which, in trays well separated from the lipoprotein, absorbed the water. After 40 hr. the dried material (moisture content approximately 4.5%) was removed, thoroughly mixed in a blender at 0°, and sampled for dry weight determination (vacuum oven at 70°).

For storage at constant relative humidity (R.H.) samples of the dried lipoprotein (approx. 0.8 g.) were held at 37° in shallow aluminium dishes in evacuated vessels over P_2O_5 (0% R.H.) and over solutions of H_2SO_4 producing known water vapour pressures. In earlier experiments a preliminary equilibration with the storage atmosphere was carried out at a lower temperature, but the finely divided product of freeze-drying reached equilibrium very rapidly *in vacuo* at 37° and this precaution was subsequently omitted.

Table 1. *Effect of ether on the dispersibility of lipovitellin*

(Preparation 18, in NaCl solutions, w/v.)

pH	N in solution after 16 hr. at 3° (as % total N)					
	1% NaCl	1% NaCl-ether	5% NaCl	5% NaCl-ether	10% NaCl	10% NaCl-ether
5.0	1	1	10	12	11	13
5.5	1.5	1.5	26	34	33	36
6.0	2	2	46	69	56	77
7.0	6	6	51	78	65	88

Determination of free lipid. The degree of decomposition of the lipoprotein was measured by estimating the amount of lipid extractable with ether. NaCl (500 mg.) was added to 5 ml. portions of the suspension containing approximately 500 mg. lipoprotein and the solution extracted at 0° by shaking in a 'Microid' wrist-action shaker with ether (100 ml.) saturated with water, usually for 15 min. The ether was then separated and its lipid content determined by drying on a rotary film evaporator (Lea, Hannan & Rhodes, 1951), taking up in dry ether, filtering and redrying, first under an infrared lamp and finally to constant weight *in vacuo* at 70°.

Lipoprotein samples which had been dried were shaken with dry ether for 30 min. at 0° or laboratory temperature, filtered, and the lipid content of the extract plus washings determined as above. In a second procedure, the dried samples (500 mg.) were re-moistened with 10% (w/v) NaCl (5 ml.) and extracted as for undried samples.

Freezing, thawing and storage in the frozen state. To investigate the effects of freezing, the lipovitellin suspension was frozen as a shallow layer in thin aluminium dishes by immersion in liquid at temperatures between –1 and –183°. Thawing was by immersion in water at controlled temperatures between 0 and 37°, the colder samples being first warmed up to –6°. Lipoprotein used for investigation of changes during storage in the frozen state was frozen and thawed under conditions found to cause minimal damage, i.e. by rapid freezing at low temperature and rapid thawing at 37°. The frozen material was held in closed vessels over ice to prevent desiccation.

Drying and storage in the dried state. For freeze-drying, the lipovitellin suspension (approx. 10% solids) was rapidly frozen in shallow layers in thin metal trays by immersion in solid CO_2 -ether at –60 to –70°. The trays were then trans-

RESULTS

Some properties of lipovitellin

Solubility. Saturation with ether (0.8 ml./10 ml. solution) considerably increased the dispersing power of aqueous sodium chloride solutions (Table 1). This amount of ether was therefore used in the routine determination of solubility.

Fig. 1. Effect on the solubility of lipovitellin (preparations 18, 23, 28) of (a) NaCl concn. at pH 6.8 and (b) of pH at 15% (w/v) NaCl. Temp. 0°; total concn. of lipoprotein 2% (w/v); 0.8 ml. ether present/10.6 ml. solution.

The effects of varying ionic strength at constant pH, and of varying pH at constant ionic strength are shown in Fig. 1. A concentration of 15% (w/v)

sodium chloride and a pH of 6.0 were usually employed in comparing the solubilities of treated samples.

Extractability of the lipid. Fresh lipovitellin suspension at pH 6.8, when dispersed by the addition of sodium chloride and extracted with ether, as described above, yielded lipid to the solvent at a slow and linear rate of approximately 3% of the total lipid present per hour (Fig. 2a). After adjustment to pH 5.2 with hydrochloric acid and standing 24 hr. at 0° a much larger proportion of the lipid was extractable with ether, 20% being removed in 30 min. and 25% in 2 hr.

Fig. 2. Extractability with ether at 0° of lipid from (a) fresh (moist) and (b) freeze-dried lipovitellin (preparation 22). A, B, extraction from 10% (w/v) NaCl containing 10% (w/v) lipovitellin; C, D, extraction from 10% (w/v) aqueous lipovitellin suspension. Anhydrous Na_2SO_4 (1.2 g./ml.) added; E, F, extraction of dry lipovitellin.

Addition of anhydrous sodium sulphate to the aqueous suspension-ether mixture, followed immediately by shaking, i.e. dehydration during extraction, resulted in a greatly increased liberation of free lipid, most of which was extracted in the first 15 min. After 2 hr. 35% of the total lipid had been liberated at pH 6.8 and 40% at pH 5.2 (Fig. 2a).

Effect of pH and of sodium chloride on extractability of the lipid. To investigate further the effect of pH on the extractability of the lipid, samples of lipovitellin suspension (approx. 10% solids) were dispersed by the addition of sodium chloride (10%, w/v) and the pH adjusted from the original value of 7.0 to the required figure by the slow addition of citric acid or dilute sodium hydroxide solution. After standing for 30 min. the suspensions were extracted by shaking with ether for 30 min. at 0°. The result (Fig. 3) showed that only a trace of free lipid was extractable by ether at pH values above 5.5, but that increasing amounts were extracted as the pH was reduced below this point. A sample of the suspension acidified to pH 5.2 with hydrochloric acid gave the same result as with citric acid, but

holding the acidified suspension at 0° for 16 hr. before extraction increased the proportion of the lipid extracted from 7 to 13%.

Fig. 3. Effect of pH on the extractability of the lipid of lipovitellin (preparation 28) from 10% (w/v) NaCl with moist ether at 0°. pH adjusted with citric acid and extracted after 30 min. except where indicated.

Dialysed aqueous suspensions of lipovitellin at approximately neutral pH were resistant to extraction by ether, but the addition of very small quantities of sodium chloride caused the liberation of free lipid, although the amount fell again as the concentration of sodium chloride was further increased and the lipoprotein began to dissolve (Fig. 4). It was observed that the aqueous phase showed an increased tendency to disperse in the ether and to settle less readily after shaking at sodium chloride concentrations which gave the highest yields of free lipid.

Fig. 4. Effect of NaCl on the extraction of lipid from aqueous lipovitellin (preparation 29) at pH 6.8 by shaking with moist ether (100 ml. ether/3 g. 10% (w/v) suspension) for 15 min.

Isoelectric point. Since lipovitellin is practically insoluble in water and in dilute buffers, an approximate estimate of its isoelectric point was obtained by determination of the pH at which the addition of sodium chloride neither increased nor decreased the apparent hydrogen-ion concentration (Schmidt, 1945). For this purpose, a concentrated lipovitellin

suspension (approx. 10 % solids) was adjusted to the required initial pH value by the addition of dilute hydrochloric acid, and the effect of adding a small quantity (0.021 M) of sodium chloride measured. The results (Fig. 5a) indicated an isoelectric point in the region of pH 5.50. With another preparation of lipovitellin (no. 25) additions of 0.011, 0.021, 0.043 and 0.086 M-sodium chloride gave isoelectric points at pH's 5.59, 5.53, 5.56 and 5.55.

Fig. 5. The isoelectric point of lipovitellin (preparation 26). *a*, Changes in pH produced in lipovitellin suspensions by the addition of NaCl (0.021 M); *b*, electrophoretic mobility as a function of pH in 0.0033 M-phosphate-citrate buffers.

In an alternative procedure, the determination was carried out electrophoretically using the cell perfected by McQuillen (1950) for microscopic observation of the movement of bacterial cells in a reversible electric field. Mobilities were measured for a considerable number of particles of lipovitellin over a range of pH values in very dilute citrate-

phosphate buffer. These values plotted against pH indicated zero mobility in the region of pH 5.57 (Fig. 5b).

Stability at 37°. Fresh lipovitellin suspension decomposed comparatively rapidly at 37° whether the criterion was loss of solubility or increase in ether-extractable lipid (Table 2). Increasing the total salt present from 0.9 % (w/v) sodium fluoride (used as a bacterial inhibitor) to 10 % (w/v) by the addition of sodium chloride had no very marked effect on the stability of the lipoprotein. The rate of liberation of lipid from all three preparations was somewhat greater than previously reported (Lea & Hawke, 1951), but the total lipid contents were also considerably higher.

Effect of freezing and thawing, and of storage in the frozen state

In preliminary experiments, more damage was found to result from the freezing and thawing of lipovitellin suspensions than from freeze-drying. Attention was therefore first directed to the thawing process. Table 3 shows that, after freezing at -65°, solubility was best preserved by thawing as rapidly as practicable in water at 37°. Under these conditions changes were small at pH 6.8, but were still large at pH 5.2 with the lipovitellin preparation used. When rapid thawing was standardized, the lipoprotein suffered less loss of solubility the lower the freezing temperature, i.e. the more rapid the freezing (Table 4).

The proportion of the total lipid extractable with ether after freezing and thawing appeared to be largely independent of the temperature of freezing,

Table 2. *Stability of fresh lipovitellin suspensions and of remoistened, freeze-dried lipovitellin at 37°*

Preparation no.	pH	Composi- tion of solvent*	Days at 37°							
			Lipovitellin			Freeze-dried lipovitellin				
			0	2	5	0	1	2	5	
			Percentage of total N soluble							
22	6.8	A	93	52	52	87	—	47	39	
22	6.8	B	93	50	50	87	—	39	37	
23	6.8	A	97	52	—	—	—	—	—	
20	6.8	A	—	—	—	94	62	53	40	
22	5.2	A	89	38	38	60	—	17	12	
22	5.2	B	89	28	28	60	—	12	10	
23	5.2	A	87	39	—	—	—	—	—	
20	5.2	A	—	—	—	94	30	19	—	
			Percentage of total lipid free†							
22	6.8	A	1.5	11	14	3	—	17	26	
22	6.8	B	1.5	10	13	3	—	10	16	
22	5.2	A	16	23	32	22	—	25	33	
22	5.2	B	15	19	27	22	—	23	26	
23	5.2	A	15	20	—	—	—	—	—	

* A, 0.9 % (w/v) NaF; B, 0.9 % (w/v) NaF + 9.1 % (w/v) NaCl.

† Wet extraction method.

and averaged 2% at pH 6.8 and nearly 40% at pH 5.2, part of the latter being attributable to acidification rather than to freezing. The amount of free lipid produced by freezing lipovitellin at pH 6.8 varied appreciably in different preparations (e.g. 2, 8, 14 and 9% in preparations 21, 23, 24 and 25 respectively), although altering the temperature, and hence the rate of freezing still showed no marked effect.

Table 3. *Effect of speed of thawing (after freezing rapidly at -65°) on the solubility of lipovitellin (preparation 22)*

Thawing temperature ($^{\circ}$)	Thawing time (min.)	Solubility (%) [*] after freezing and thawing at	
		pH 6.8	pH 5.2
Not frozen	—	92.6	88.8
37	0.7	88.3	53.7
5.4	3.7	84.7	43.9
1.0	12	86.2	47.6
0.0	35	71.8	37.1

^{*} In 15% (w/v) NaCl—ether at pH 6.

Table 4. *Effect of freezing at various temperatures on lipovitellin*

(Preparation 21; thawing at 37° .)

pH	Temp. of freezing ($^{\circ}$)	Solubility (%)	Percentage of total lipid free
6.8	-3	55	2
6.8	-7	55	2
6.8	-20	66	2
6.8	-65	63	—
6.8	-183	74	1
5.2	-3	17	40
5.2	-7	19	38
5.2	-20	21	36
5.2	-65	36	36
5.2	-183	40	42

Fig. 6. Effect of storage temperature on the development of insolubility in frozen lipovitellin at pH 6.8 (○) and pH 5.2 (●). Preparation 23.

To isolate the effects of storage in the frozen state a lipovitellin suspension was frozen at -65° , stored at various constant temperatures below 0° and thawed rapidly in water at 37° . The results (Fig. 6) show that solubility decreased rapidly at first at

both pH's and at all four storage temperatures, and thereafter decreased more slowly at rates which varied with the temperature from practically zero at -29° to a rate at -3° which produced almost complete insolubility within 3 months. At pH 6.8, very little free lipid was split off during 3 months at -29° and only slightly more at -20° . The liberation of lipid however was considerable at -10° and

Fig. 7. Effect of storage temperature on the liberation of lipid from lipovitellin suspension (pH 6.8) frozen at -65° . (Thawed at 37° ; ○, preparation 23; ×, preparation 25.)

particularly at -3° . At all four temperatures, most of this change occurred during the first 10 days of storage (Fig. 7). At -3° the appearance of the samples changed from white to translucent, presumably owing to the growth of the larger ice crystals at the expense of the small.

Effect of freeze-drying

Solubility. Freeze-drying of lipovitellin suspensions by the described procedure had a variable effect on the dispersibility of the lipoprotein in 15% (w/v) sodium chloride-ether at pH 6 (Table 5), some preparations apparently being more susceptible to damage than others. Solubility in all cases was higher when drying was at pH 6.8 than at pH 5.2, and the dried product in both cases could be held at -20° for some weeks with little further change.

Extractability of the lipid. A considerable proportion of the lipid of freeze-dried lipovitellin was extractable with ether. Four preparations of the lipoprotein freeze-dried at pH 6.8 yielded 24, 24, 30 and 20% (average 24.5%) of extractable lipid. At pH 5.2 the yields were slightly lower, namely 22, 18, 26 and 18% (average 21%). Extraction was almost complete after 15 min. (Fig. 2b). If, however, the material dried at pH 6.8 was re-moistened with water before extraction, very much smaller quantities of lipid passed into the ether, and the proportion of the total lipid removed in 2 hr. (8–9%) was only slightly greater than from undried lipo-

Table 5. *Effect of freeze-drying on the solubility of lipovitellin*

pH of suspension	Treatment	Preparation no.			
		20	22	23*	24*
		Solubility (%) in 15 % (w/v) NaCl-ether at pH 6			
6.8	Fresh	94.7	92.6	96.7	94.4
	Freeze-dried	94.2	86.8	76.5	70.3
	Freeze-dried and held 13 days at -20°	94.1	—	—	—
5.2	Fresh	94.7	88.8	87.3	88.1
	Freeze-dried	94.0	60.2	55.5	56.5
	Freeze-dried and held 13 days at -20°	93.8	—	—	—

* Sucrose present during the preparation (cf. p. 105).

vitellin similarly treated (Fig. 2b). With another preparation the difference between the fresh material and that re-moistened after drying at pH 6.8 was still smaller. On the other hand, extraction of re-wetted material which had been dried at pH 5.2 yielded even more lipid than did dry extraction, the limiting amount reached (25%) being the same as that liberated from the undried lipoprotein.

Fig. 8. The vapour pressure isotherm of freeze-dried lipovitellin (preparation 20) at 37° . Lipid content 38.7% of dry weight.

Stability after re-moistening. A comparison of the behaviour on keeping at 37° of lipovitellin and of the same material after freeze-drying and re-moistening to the original water content is given in Table 2. Although some decomposition frequently resulted from the drying process itself, little if any increase in susceptibility to further decomposition on storage of the moist material at 37° was apparent.

The vapour-pressure isotherm of freeze-dried lipovitellin. The relationship between the equilibrium relative humidity and the moisture content of freeze-dried lipovitellin as determined by drying to constant weight *in vacuo* at 70° is given in Fig. 8. The isotherms at pH's 6.8 and 5.2 were indistinguishable. The storage experiments with dried material were carried out at constant relative humidities.

Effect of storage in the dried state

Solubility. Freeze-dried lipovitellin in equilibrium with atmospheres of high relative humidity, i.e. at high moisture contents, became insoluble extremely rapidly, much more rapidly, in fact, than when suspended in water or dispersed in sodium chloride solution (Table 2). In a completely dry atmosphere the rate of change was very slow, while atmospheres of intermediate composition produced intermediate rates of change (Fig. 9). In all cases, insolubility developed considerably more rapidly in the material adjusted to pH 5.2 before drying than in that adjusted to pH 6.8. A perceptible lag or induction period noticeable with certain of the samples which became insoluble at intermediate rates was not detectable under conditions producing more rapid deterioration.

Extractability of the lipid. Fig. 10 shows that, qualitatively, the effects of storage in moist and dry atmospheres on the liberation of free lipid (as estimated by extraction of the 'dry' lipoprotein) were similar to the effects on solubility, the changes being very rapid at high humidities and very slow at low humidities. Quantitatively, however, the parallelism was far from complete. For example, the liberation of free lipid seemed always to be complete within 1 day at 37° , and samples held at 10% R.H. for as long as 45 days still gave the same free lipid values as at 1 day, i.e. 31.5 and 28% respectively at pH's 6.8 and 5.2. The solubility data at pH 6.8 and 70 or 85% R.H., and at pH 5.2 and 30% R.H., on the other hand, showed an 'induction period' of 1 day during which very little loss of solubility occurred, although thereafter the change was rapid and speedily approached a limiting value. Furthermore, at intermediate R.H. values, such as 70% R.H. at pH 6.8 and 70, 55 and 30% R.H. at pH 5.2, the loss of solubility during storage was relatively very much more marked than the liberation of lipid (Figs. 9 and 10).

The free lipid estimations in the storage experiments were normally carried out by extraction of

the solid ('dry') lipoprotein with ether, but extraction by the 'wet' method, i.e. after redispersion in sodium chloride solution, showed generally similar changes in an experiment in which the two methods were tested against one another on a

decrease in rate at high humidities with any certainty. The cause of the darkening on storage has not yet been ascertained: it may be due to autoxidation of the lipid, or to a 'browning' reaction of the amino-aldehyde type, or to both.

Fig. 9. Effect of variation in moisture content (equilibrium R.H. indicated) on the development of insolubility in freeze-dried lipovitellin (preparation 20).

Fig. 10. Effect of variation in moisture content (equilibrium R.H. indicated) on the liberation of free lipid (dry-extraction method) from lipovitellin (preparation 20).

different (and apparently more stable) specimen of freeze-dried lipovitellin which had been stored at 85% R.H. (Table 6).

Colour. The slightly 'off-white' colour of freshly freeze-dried lipovitellin darkened on storage, the change being greatly accelerated by an acid pH and a high relative humidity (Table 7). The rate of discoloration appeared to be maximal at about 85% R.H., but the data are too few to establish the

DISCUSSION

A troublesome feature of this work on the freezing and drying of lipovitellin has been a certain lack of exact reproducibility in the behaviour of preparations made in apparently the same way when submitted to apparently similar freezing and drying treatments. It is not known whether these differences derived from the eggs, which were collected

from various sources and at various seasons, or from chance variations in technique. Despite this limitation, the results have some interest both from the theoretical viewpoint of the properties and structure of lipoproteins and from the more practical one of the behaviour of these substances in biological materials preserved by freezing or drying.

State of the lipid in lipovitellin. Fresh lipovitellin dispersed in 10% (w/v) sodium chloride solution gave up lipid to ether only very slowly at pH values above 5.5, but up to one-third of the lipid could be extracted rapidly from more acid salt solutions (Fig. 3) in which the lipoprotein was much less soluble. A similar proportion of the lipid could also be obtained at neutral pH from suspensions of lipovitellin in sodium chloride solutions too weak to disperse it. It seems reasonable to suppose that the

extraction, and even more (30–35%) if the moist lipovitellin were dried by sodium sulphate during extraction (Fig. 2), it would appear that water has some function in stabilizing the union of the more loosely held portion of the lipid with the remainder of the complex.

To this extent, therefore, lipovitellin seems to be decomposed by drying. On the other hand, when lipovitellin, which has been freeze-dried at pH 6.8, is re-wetted, it is found to retain most of its solubility and to have recovered most of its capacity for withholding its lipid from ether: at this pH, at least, decomposition produced by drying seems to be largely reversible. At pH 5.2 the loss of solubility on freezing or drying was usually much more marked than at pH 6.8, and the lipoprotein lacked resistance to ether even before freezing or drying.

Table 6. *Liberation of free lipid from lipovitellin (preparation 24) stored at 37° and 85% R.H., as measured by 'dry' and 'wet' extraction methods*

Stored at 37° (days)	Free lipid (as % total lipid)					
	pH 6.8			pH 5.2		
	0	1	6	0	1	6
'Dry' extraction	28	38	39	25	33	34
'Wet' extraction	4	20	25	26	34	35

Table 7. *Effect of pH and moisture content on the discoloration of freeze-dried lipovitellin at 37°* (preparation 20)*

pH	Time stored (days)	Equilibrium relative humidity (%)					
		0	30	55	70	85	92
6.8	1	—	1.5	1.4	1.4	1.4	1.5
	4	1.4	1.4	—	1.3	1.4	1.5
	10	1.6	1.5	1.4	1.5	1.8	1.8
5.2	1	1.4	—	1.4	1.8	1.8	1.7
	4	1.6	1.7	1.6	2.0	3.5	2.5
	10	—	1.6	1.7	—	4.5	3.9

* Initial colour, 1.4 R + Y Lovibond units.

lipids of lipovitellin in 'solution' are inaccessible to ether through the whole lipid molecules, or at least their lipophilic portions, being orientated towards the interior of the lipoprotein molecule, away from the water surface. The extraction of a considerable amount of lipid from the moist particle in suspension in the presence of insufficient sodium chloride to disperse it, but not from suspension in pure water (Fig. 4), is difficult to account for, and may be due to some mechanical factor such as more intimate contact between the aqueous and ether phases as indicated by the slower separation of the dilute salt solution from the ether after shaking. There was a marked similarity between the amounts of lipid liberated under these conditions and by drying.

Since 15–20% of the lipid was extractable within 10 min. if the lipoprotein were freeze-dried before

Under most of the conditions investigated the amount of free lipid liberated tended to rise towards some limiting value which could be anything from 10 to 50% or even more, a circumstance which seemed to indicate a far from uniform strength of binding between different fractions of the lipid and the protein. Tayeau (1944) believes that neutral lipid is mainly bound to the phospholipins, and that phospholipins form the link between neutral lipid and protein. With such a structure, it might be possible to break selectively the protein-lipid and lipid-lipid associations in the lipoprotein, and Delsal (1949) has already found that 'delipidation' by anhydrous ether at -70° according to the method of McFarlane (1942) appears to extract only cholesterol from blood serum, the phospholipin-protein 'cénapses' remaining intact. An investigation of the

chemical nature of the lipid of lipovitellin and of the fractions removed by various physical and chemical treatments is now being undertaken.

Effect of freezing. Freezing and thawing tended to destroy the solubility of lipovitellin, the effect being greater at pH 5.2 than at pH 6.8. The damage could be reduced by freezing rapidly at low temperature and by thawing rapidly (Table 3), and there was no sign of increasing loss of solubility as more water was frozen out by lower freezing temperatures—in fact, the reverse tended to be the case (Table 4). When stored in the frozen state the lipoprotein decomposed at an appreciable rate even at low temperatures, and the difference between -20 and -29° could readily be detected after 2 or 3 months (Figs. 6 and 7). Obviously, the lipoproteins are among the constituents of biological materials most susceptible to decomposition during storage.

In freezing mammalian and fish muscle Moran (1935) and Reay (1933) have drawn attention to the rapid denaturation of the protein which occurs at temperatures just below the freezing point of the muscle, and particularly in the region of -2 to -4° , and Finn (1932) has attributed these results to the increased concentrations of muscle salts and hydrogen ions which develop in the liquid phase of the material at these 'critical' temperatures when a large proportion of the water freezes out. These natural muscle systems, however, are not comparable with the dialysed protein suspensions used in the present work, in which the concentration of salts is very low. Comparison with the experiments of Moran (1925), who was able to freeze egg yolk rapidly in liquid air and thaw it rapidly in warm mercury without the serious loss of fluidity which occurred if freezing or thawing were slow, is similarly complicated by the presence of salts in the whole yolk. Our results on the effect of pH on the stability of lipovitellin suggest that damage could easily be produced by a depression of the natural pH of the yolk (6.1–6.2) such as could be produced by concentration of the salts during freezing.

Effect of drying. Lipovitellin could usually be freeze-dried at pH 6.8 with but little decomposition, and one sample was dried even at pH 5.2 without loss of solubility, although deterioration at this latter pH was usually marked. Lipovitellin therefore appears to be more stable than the lipoproteins of human plasma (Gurd, Oncley, Edsall & Cohn, 1949; Oncley, Gurd & Melin, 1950).

The results of storage in the dried state were particularly interesting in showing a high degree of stability at very low moisture contents and a rapid rate of deterioration at high moisture contents, with intermediate values at intermediate moisture contents (Figs. 9 and 10). Deterioration in the moist solid was, in fact, much more rapid than in the fully wetted state or in 'solution'. This result, while in

keeping with the non-harmful effect of freezing at very low temperatures, is in apparent conflict with the theory that water forms an essential part of the lipoprotein complex. It could perhaps be conceded that, in a union split by drying, the components might be unable to undergo further change under very dry conditions and would combine again on rehydration. The failure of ether extraction to remove increased amounts of lipid from the dried material after storage at low humidities, whilst readily doing so from dried material after storage at high humidities (Fig. 10), however, could more easily be explained by a theory of increasing liberation of lipid due to progressively greater denaturation at increasing moisture contents of the protein with which it had been combined. On the other hand, loss of solubility and liberation of free lipid do not always bear the same relationship to one another (cf. p. 110), and the assumption would be necessary that in some cases an appreciable interval elapses between the denaturation which liberates the lipid and the aggregation which results in the loss of solubility. Even this explanation does not cover the few cases already referred to (p. 110) where a marked loss of solubility accompanied a relatively minor liberation of lipid, i.e. where denaturation appeared to precede splitting. The precise nature of the connexion between loss of solubility and liberation of free lipid has not, therefore, been established.

From the viewpoint of practical dehydration and storage the desirability of drying to low ultimate moisture contents and, during drying, of passing rapidly and at low temperature through the range of critical moisture contents where deterioration is rapid can readily be seen from Figs. 9 and 10.

SUMMARY

1. The effects of freezing and drying lipovitellin and of storage in the frozen and dried states have been investigated.

2. Decomposition of the lipoprotein was minimized by rapid freezing and thawing, and was not increased by very low freezing temperatures. Further changes occurred during storage in the frozen state at temperatures down to -29° , and at -3° the lipoprotein became completely insoluble.

3. Lipovitellin could be freeze-dried at approximately neutral pH with but little decomposition, and was then comparatively stable at low storage temperatures, or even at 37° if very dry. In equilibrium with atmospheres of high relative humidity the moist solid decomposed much more rapidly than either the dry solid or the fully moistened or dispersed lipoprotein.

4. About one-quarter of the lipid of freshly freeze-dried lipovitellin was readily extractable with ether, but on moistening (at neutral, but not at acid,

pH) most of this lipid again became extractable only with difficulty.

5. The parallelism between the two criteria of decomposition used, namely dispersibility in sodium chloride solution-ether and extractability of the lipid with ether, was not complete.

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The Effects and the Fate of Malononitrile and Related Compounds in Animal Tissues

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It is generally accepted that the toxicity of aliphatic nitriles in the animal body is mainly due to a gradual release of cyanide. The evidence on which this view is based consists (a) in the similarity of the symptoms of intoxication to those of cyanide poisoning, with due consideration of the different rates at which cyanide is liberated from different nitriles, (b) in the antidotal effect of thiosulphate, and (c) in the increased excretion of thiocyanate (cf. Hunt, 1923). The second and third facts are to be attributed to the action of the enzyme rhodanese which catalyses the reaction (Lang, 1933):

The formation of cyanide from nitriles has, however, not yet been demonstrated *in vitro*. Lang (1933) investigated the formation of SCN^- from acetonitrile and propionitrile by minced tissues with negative results. Warburg (1911; Warburg & Wiesel, 1912), who found an inhibition of the respiration of avian erythrocytes and of the fermentation of yeast cells by high concentrations of mononitriles, came to the conclusion that this was an unspecific 'narcotic' effect.

Recently, Hydén & Hartelius (1948) described an effect of malononitrile which they could not reproduce with cyanide. Using the microspectrographic method they found, in experiments on rabbits, an increase in the concentration of cytoplasmic protein and nucleoprotein of ganglion cells 1-48 hr. after an injection of malononitrile. Their observations are at variance with the results of Hicks (1950), Liu (1951) and Reale & D'Argenio (1951) using histological techniques, and with those of Samuels, Boyarsky, Gerard, Libet & Brust (1951) using chemical methods. Nevertheless, the general importance of the claims of Hydén & Hartelius and the particular interest arising from their recommendation of malononitrile therapy in mental diseases suggested an investigation into the effects of malononitrile and other nitriles on the metabolism of tissue slices, and into the correlation of any such effects with the release of cyanide. As Heymans & Masoin (1897) have shown, the toxic action of malononitrile is fast; it seemed therefore well suited for short *in vitro* experiments.

Another class of compounds from which cyanide is gradually liberated, in the body as well as in