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Lipovitellin

3. THE EFFECT OF SUGARS ON THE STABILITY OF FREEZE-DRIED LIPOVITELLIN

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The effects of freezing and drying lipovitellin and of storage in the frozen and dried states have already been investigated (Lea & Hawke, 1952*a-c*). The present paper describes attempts to control the deteriorative changes which occur during and after freeze-drying by the addition of carbohydrate before freezing.

The value of sugars, particularly lactose, for the stabilization of proteins during drying has previously been reported (Brosteaux & Eriksson-Quensel, 1935). Sucrose and glycerol have been shown to inhibit the heat coagulation of rabbit serum and of egg albumin (Beilinson, 1929), and various hexoses and pentoses, including glucose, fructose, mannose, arabinose and xylose, have been found to protect proteins from heat denaturation in aqueous solution as measured by the liberation of SH groups (Ball, Hardt & Duddles, 1943). Moran (1925) found that added sugars prevented the increase in viscosity which normally occurs in egg yolk during freezing and thawing, and sucrose and glycerol are used commercially for this purpose. The presence of added carbohydrate, especially lactose or in less degree sucrose, has been found to retard loss of solubility and loss of aerating power of spray-dried egg during storage, but glucose and particularly arabinose accelerated these changes (Brooks & Hawthorne, 1943). On the other hand, Fry &

Greaves (1951) found that glucose, lactose, sucrose, raffinose and xylose all increased the survival of bacteria during freeze-drying and subsequent storage.

For the present purpose sucrose, lactose and glucose have been selected as representative sugars, and their effects on the solubility and free lipid content of lipovitellin have been determined after freeze-drying and during subsequent storage in the 'dry' state under various conditions.

METHODS

A lipovitellin suspension containing approximately 10% solids was prepared as previously described (Lea & Hawke, 1951, 1952*c*), adjusted to the required pH, allowed to stand at 0° overnight in the presence of sugar equal in weight to the lipoprotein present, mixed, and frozen rapidly in shallow layers in thin metal trays by immersion in liquid at -60 to -70°. After reducing the moisture content of the frozen material to about 2% by freeze-drying for 2 days in the presence of P₂O₅ (Lea & Hawke, 1952*c*) the product was mixed by brief agitation in a blender at 0° and the fluffy, off-white powder stored at 37° over H₂SO₄ solutions producing the required water vapour pressures.

Lipovitellin and samples containing respectively sucrose, lactose and glucose were prepared at pH 6.8, and stored at 30, 70 and 92% relative humidity (r.h.). At pH 5.2 lipovitellin and lipovitellin-sucrose only were prepared and storage was at 70% r.h. only.

Table 1. *Effect of sugars on the initial solubility and free lipid content of freeze-dried lipovitellin*

pH of suspension	Lipovitellin alone		Lipovitellin dried with		
	Before drying	After drying	Sucrose*	Lactose*	Glucose*
		% of total N soluble			
6.8	94.8	87.6	94.3	94.3	94.1
5.2	88.0	86.1	88.4	—	—
		% of total lipid free†			
6.8	—‡	20.9	6.5	6.0	11.8
5.2	—	14.8	4.5	—	—

* Equal weight to the lipoprotein.

† Determined by dry extraction with ether. Total lipid content 36.4%.

‡ Not determined, but usually about 2%.

The methods used for examination of the fresh and stored materials have been described (Lea & Hawke, 1952c). They consisted in (a) determination of the percentage of the lipoprotein N passing into solution when 2% (w/v) lipovitellin was dispersed at pH 6.0 in 15% (w/v) NaCl saturated with ether at 0°, (b) determination of the free lipid present by extraction of the dry solid with ether at 0°, and (c) measurement of the colour of the solid by reflected light in a Lovibond-Schofield Tintometer.

RESULTS

Protective effect of sugars during drying

The lipovitellin preparation (no. 30) used for most of the experiments contained 36.4% of bound lipid. Table 1 shows that freeze-drying in the presence of any one of the three sugars gave a product in which solubility had been completely retained, whereas an appreciable loss of solubility resulted when this sample of the lipoprotein was freeze-dried alone.

Although the quantity of free lipid extracted by ether from the dry sugar-containing products was rather more in all cases than that usually obtained from undried lipovitellin suspensions it was considerably less than that released by drying in the absence of sugar; glucose, however, was appreciably inferior to sucrose and lactose in this respect (Table 1).

The water-sorption isotherm of lipovitellin and of lipovitellin-sugar mixtures

The relationship between equilibrium R.H. and the corresponding water contents of freeze-dried lipovitellin and of lipovitellin-sugar mixtures, as determined by drying for 3 hr. *in vacuo* at 70°, is shown in Table 2.

Rather similar moisture contents were observed in all three sugar-containing samples at 30 and 70%, but at 92% R.H. lipovitellin-lactose took up much less water than the other mixtures, probably owing to crystallization of the hygroscopic, amorphous, 'glass' form of the sugar to give the non-hygroscopic crystalline α -lactose monohydrate. Such a crystal-

lization has been found to occur during the storage of spray-dried skim-milk powder at high moisture contents (Henry, Kon, Lea & White, 1948). All the sugar mixtures, with the exception of this lactose sample, held more water than the corresponding sugarless controls.

Table 2. *Water contents of freeze-dried lipovitellin and of lipovitellin-sugar mixtures at various relative humidities. (Temp. 37°)*

Sugar content (% of final dry wt. added before drying)	g. water/100 g. dry material at equilibrium R.H.		
	30%*	70%*	92%†
No sugar added	2.5	10.7	24.0
50% Sucrose	3.3	14.9	49.8
50% Lactose	3.6	14.8	18.5
50% Glucose	2.9	15.5	65.3

* After 3 days at 37°.

† After 1 day at 37°.

Equilibrium with the storage atmosphere was reached in less than 24 hr. at 37°, and thereafter the weight remained approximately constant or, particularly at the higher moisture contents, tended to decrease. This decrease in weight may be associated with partial denaturation since Barker (1933) has pointed out that at a given R.H. heat-denatured egg albumin is in equilibrium at a lower moisture content than is native egg albumin.

Effects during storage in the 'dry' state

Solubility. Fig. 1 shows that lipovitellin dried in the presence of sucrose from suspension at pH 6.8 retained its solubility better during subsequent storage than did lipovitellin alone. This was true at all three humidities, an approximate estimate of the ratio of the times required for equivalent deterioration being 2-3:1 at 30% R.H., 3-4:1 at 70% R.H. and 4-5:1 at 92% R.H. Similar protective effects were observed at pH 5.2 although under these more acid conditions the change was approximately four times as rapid (Fig. 2).

At 30 and 70 % R.H. lactose also showed a marked protective action against loss of solubility which, however, was somewhat less than that of sucrose. At 92 % R.H. no appreciable protection was afforded by the presence of lactose and the mixtures closely followed the control (Fig. 1).

The presence of glucose, rather than inhibiting, promoted the development of insolubility in freeze-dried lipovitellin. Under the driest storage conditions when the control lipoprotein was comparatively stable this effect was very apparent, but it tended to disappear under very moist conditions

been made. At 92 % R.H., however, there was considerable liberation of lipid from the control lipovitellin (as well as from lipovitellin-lactose, anomalous at this R.H.), whilst added sucrose exerted a marked protective action.

Colour. The brown discoloration, which develops during the storage of freeze-dried lipovitellin and which is more marked at pH 5.2 than at pH 6.8 (Lea & Hawke, 1952c), was modified by the presence of sugars. The major factors involved appear to be the moisture content of the system and the nature of the sugar.

Fig. 1. Effect of sugars on the development of insolubility in lipovitellin freeze-dried from suspension at pH 6.8 during storage at different relative humidities. C, control without sugar; S, sucrose; L, lactose; G, glucose. Sugar: lipoprotein ratio = 1:1.

Fig. 2. Effect of sucrose on the development of insolubility and of free lipid in lipovitellin freeze-dried from suspension at pH 5.2 and stored at 70 % R.H. C, control without sugar; S, sucrose.

when the control material itself lost solubility extremely rapidly. It is possible that determinations of solubility at an earlier stage of the reaction than that represented by 24 hr. at 37° might have shown acceleration by glucose even at high humidities but equilibration with the storage atmosphere would not have been complete with very short exposures.

Extractability of the lipid. Figs. 2 and 3 show that at 30 and 70 % R.H. comparatively little increase in extractable lipid occurred during storage, the major effect of the sugars being confined to the protective action during drying to which reference has already

Fig. 3. Effect of sugars on the liberation of free lipid from lipovitellin freeze-dried from suspension at pH 6.8 during storage at different relative humidities. C, control without sugar; S, sucrose; L, lactose; G, glucose.

Fig. 4. Effect of sugars on the discoloration of lipovitellin freeze-dried from suspension at pH 6.8 (or pH 5.2) during storage at different relative humidities. C, control without sugar; S, sucrose; L, lactose; G, glucose.

At 30 % R.H. changes were very slow and only the glucose sample showed a slight increase in colour (Fig. 4). At 70 % R.H. and pH 6.8 the control and the lactose samples again showed practically no change while the glucose sample darkened considerably and the sucrose sample slightly. At 92 % R.H. browning was very rapid with glucose and was quite marked with sucrose. Lactose presumably crystallized at this high R.H. (since the moisture content of the lactose preparation was even less than that of the control), and the two samples darkened at the same comparatively slow rate.

DISCUSSION

The protective effect shown by all three sugars during the freeze-drying of lipovitellin, whether measured by loss of solubility or by the appearance of free lipid, is in parallel with the results of Ball *et al.* (1943) on the heat denaturation of proteins in aqueous solution. Protection during drying, however, seems to be a rather non-specific phenomenon, witness for example the stabilization of the α -lipoprotein of human plasma by serum albumin during drying (Cohn, Surgenor, Schmid, Batchelor & Isliker, 1952).

The results of the storage experiments in the 'dry' state show a marked protective effect of sucrose on the solubility of lipovitellin under all conditions, a somewhat less marked protective action of lactose and an acceleration of deterioration by glucose. These observations are similar to those reported by one of us (Lea, 1948) on the reactions of these sugars with 'dry' dialysed milk protein, where it was concluded that two mechanisms are probably involved, namely (a) a general retardation of the development of insolubility under the influence of relatively high concentrations of carbohydrate, and (b) an acceleration induced by reaction between the reducing group of the sugar and the protein free amino groups. Sucrose could then be considered as behaving entirely according to mechanism (a), glucose (and more particularly aldopentoses) predominantly according to mechanism (b) and lactose according to both. The fact that the reaction between reducing sugars and protein amino groups is greatly favoured by the 'dry' state and hardly occurs in dilute aqueous solution at non-alkaline pH would suggest an explanation for the opposite effects of glucose reported in wet and dry systems. The work of Fry & Greaves (1951) on preservation of the viability of freeze-dried bacteria with the aid of sugars might seem to provide an exception, but here the nature of the limiting changes involved in death of the bacteria are not known and the authors themselves appear to regard the sugars merely as hygroscopic substances which retain a vitally important residual concentration of water in the system.

When changes in the extractability of the lipid during storage are considered, it can be seen that the incomplete nature of the correlation between the two main criteria of decomposition used, i.e. low solubility and high free lipid content, which was noted previously (Lea & Hawke, 1952c) has become still more apparent in the present work. Thus, during storage at 30% R.H. little or no increase in

free lipid occurred in any of the samples (Fig. 3), whereas very marked losses of solubility occurred in some of them (Fig. 1). At 70% R.H. too, the very small increases in the free lipid contents of the control and glucose samples contrast with the almost complete loss of solubility in salt solution. It would seem from these results that, contrary to the view of Gurd, Oncley, Edsall & Cohn (1949) on plasma lipoproteins, lipid is not necessarily liberated from its association with protein when the latter is denatured, although there is often a marked parallelism between the two phenomena (Lea & Hawke, 1952c).

In the development of discoloration during storage glucose showed a similar acceleration to that previously observed with casein (Lea, 1948; Lea & Hannan, 1949) and at very high moisture contents even sucrose enhanced browning.

From these experiments there seems no doubt that some, at least, of the observed protective action of sugars against the deteriorative changes in dried egg (Brooks & Hawthorne, 1943) can be attributed to the protection afforded to its lipoprotein constituent.

It should be pointed out that of the three R.H.'s studied the two lower are the more interesting from the viewpoint of practical preservation by drying, 30% R.H. being a common value for a dried product and 70% R.H. a common atmospheric humidity.

SUMMARY

1. The effects of sucrose, lactose and glucose on the stability of lipovitellin during freeze-drying and during subsequent storage in the 'dry' state at various moisture contents have been investigated.
2. All three sugars prevented the small loss of solubility which occurred in the untreated lipoprotein during freeze-drying.
3. Sucrose and in less degree lactose also retarded loss of solubility during storage, but glucose accelerated this.
4. The sugars showed some protection of the lipid-protein association, but marked losses of solubility with little or no accompanying increase in free lipid were frequently observed.
5. Glucose increased discoloration during storage, particularly at high moisture contents.

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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