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The Relative Rates of Oxidation of Lipovitellin and of its Lipid Constituent

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Unsaturated lipids in general, and phospholipins in particular, are known to undergo oxidation when exposed to the atmosphere, and such changes are liable to occur whenever lipid-containing substances are handled. The recent observation by Dam and co-workers (Dam, 1944 *a, b*; Dam & Granados, 1945 *a, b*) that fat peroxides and a yellow-brown discoloration can develop in tissues *in vivo*, particularly when the diet is deficient in tocopherols (which act as anti-oxidants) and rich in highly unsaturated fatty acids, lends added biochemical interest to a process hitherto considered mainly of importance in extracted lipids and 'dead' tissues.

Lipids, which in the isolated state are very susceptible to attack by oxygen, often show in the tissue an astonishing resistance; linseed oil, for example, remains unoxidized for long periods in the harvested flax-seed. Although the difference is probably connected with the fact that inhibiting substances such as phenolic anti-oxidants are only in part extracted with the lipid, the further possibility exists that the more reactive lipids may be stabilized against oxidation by existing in combination with other constituents of the tissues. Triglyceride fat, and particularly that present in the large fat storage depots, is mainly free, whilst much of the phospholipin occurs in combination with protein or (in less degree) carbohydrate in the cells. It seemed of interest, therefore, to compare the rates of oxidation, both in the presence and absence of catalysts, of phospholipin (*a*) in combination with protein, and (*b*) in the free state, either alone or admixed with the protein.

Lipoproteins in general tend to be ill defined, non-crystalline, labile substances, difficult to isolate, characterize and handle. Lipovitellin from egg yolk was chosen for the present work because its comparatively high stability and low degree of unsaturation favoured separation in an undecomposed and

unoxidized state. Since the autoxidation of lipids can be greatly accelerated by exposure to light or by the presence of trace metal and other contaminants, or retarded by substances which function as anti-oxidants (inhibitors), the technique employed included precautions against interference by such factors.

EXPERIMENTAL

Preparation of lipovitellin from egg yolk

The method used was essentially that of Chargaff (1942), with modification where necessary to overcome the intractable emulsions encountered in the original procedure. Batches of 48 eggs, not more than 24 hr. old, were washed in 2% lysol and in ethanol, which was burned off before breaking. The yolks, after removal of the membrane and chalazae, were mixed with an equal volume of water and extracted by shaking five or six times with 800 ml. peroxide-free ether until the extract was practically colourless. Saturated NaCl solution (520 ml.) was then added to dissolve the lipoprotein, and the solution was dialysed through cellulose, first into water (16 l.) for several hours and then overnight into water (80 l.). All operations were carried out at 0–3° with precooled solvents and equipment. The very large quantities of water required for dialysis were prepared by mixed bed de-ionization of the laboratory distilled water supply. Cellulose casing was found to contain considerable quantities of water-soluble material and was thoroughly extracted before use.

The crude lipoprotein which separated was centrifuged, washed twice with 800 ml. water, suspended in 560 ml. water and shaken with 800 ml. ether. No ether could be separated at this stage, and 240 ml. saturated NaCl solution were added and the whole dialysed. During dialysis an ether layer separated and was discarded before centrifuging the protein. The processes of solution by NaCl, reprecipitation by dialysis, washing and extraction with ether were repeated four times, with omission of the ether extraction on the last occasion. The lipoprotein suspension obtained was held at 0° in an atmosphere of N₂, and its solids content determined by drying to constant weight *in vacuo* at 70°.

Fevold & Lausten (1946), by direct centrifugation of diluted egg yolk in a Sharples centrifuge at 40,000 rev./min., separated a solid crude 'lipovitellin' fraction which, after purification by Chargaff's method, contained 16-18% lipid. From the residual emulsion by a similar purification they recovered a product of higher lipid content (36-41%) which they termed 'lipovitellin'. In our hands separation by the Sharples centrifuge at 24,000 rev./min. (the high-speed turbine head was not available) gave a lipovitellin fraction containing only about 13% of lipid. It therefore seems probable that lipoproteins of egg yolk range widely in fat content, and 'lipovitellin' and 'lipovitellin' are probably not well defined entities. The lipoprotein obtained by the modified Chargaff procedure described above contained 22-30% of lipid and was considered suitable for the present work.

The ethanol-soluble lipid content of lipovitellin. In preliminary attempts to determine the total lipid content of the aqueous lipovitellin cream by extraction with ethanol and ether, before or after ordinary drying, trouble was encountered with emulsification or incomplete extraction. In the method finally adopted, the lipovitellin suspension (approx. 10% solids) was freeze-dried and extracted in a Soxhlet apparatus with hot ethanol for 4 hr. The extract was evaporated in a rotary film vacuum evaporator (Lea, Hannan & Rhodes, 1951), taken up in dry ether, filtered, evaporated under an infrared lamp and dried in a vacuum oven at 70°. Typical results, compared with those obtained by the repeated cold extraction procedure used by Fevold & Lausten, are given in Table 1.

Table 1. *Determination of total lipid content of lipovitellin*

Temp. of ethanol (°)	Time of extraction in Soxhlet apparatus (hr.)	No. of separate 30 min. extractions	Lipid extracted (dry wt. basis) (%)
75	2	—	25.0
75	4	—	25.7
75	6	—	25.7
Approx. 20	—	1	19.7
Approx. 20	—	2	22.1
Approx. 20	—	7	25.2

Determination of free lipid. For the estimation of free lipid present as a contaminant or produced by decomposition of the lipoprotein, it was necessary to extract the aqueous lipoprotein solution or suspension with ether. Under most conditions this gave rise to intractable emulsions, but by extracting 3 ml. of a solution containing approx. 0.5 g. lipoprotein and 0.3 g. NaCl with successive portions (100 ml.) of ether saturated with water, a satisfactory separation was achieved. 'Wrist-action' agitation in a 'Microid' shaker served to mix the phases.

When free lipid was present, it was very largely extracted in 10-30 min. at 0-3°, but prolonged extraction in this way showed that the lipovitellin preparations yielded virtually no free lipid until it slowly became soluble owing to decomposition or denaturation (Fig. 1).

Splitting with ethanol

For comparison of the rate of autoxidation of lipid combined in the lipoprotein with that of the free lipid in presence of the protein, it was necessary to split the lipoprotein complex with minimal damage to either component, and then to remove completely any agent used for splitting. This was first attempted *in situ* without gross separation of the two components, by freeze-drying the lipovitellin suspension (4 g. containing approx. 0.5 g. dry material), just moistening with absolute ethanol (2 ml.), holding for 4 hr. at the selected temperature and removing the ethanol by vacuum evaporation at the same or a lower temperature. Residual traces of ethanol were removed by addition of water and freeze-drying. Treatment with ethanol at 25° or below was found to render less than half the total lipid extractable with ether, and even ethanol at 75° failed to liberate more than 60% of the lipid (Fig. 1). Apparently, mechanical removal of the lipid as it is liberated is essential for complete splitting of the lipoprotein complex by ethanol.

Fig. 1. Effect of ethanol at various temperatures on the stability of lipovitellin. A, undried; B, freeze-dried; C, dried and treated with ethanol at -7°; D, dried and treated with ethanol at 10°; E, dried and treated with ethanol at 25°; F, dried and treated with ethanol at 75°.

Separation of the lipid from the protein. The efficiency of solvent extraction at temperatures between -28° and +25° was investigated by repeatedly extracting (with shaking) freeze-dried lipovitellin with ethanol or ethanol-ether,

Table 2. *Extraction of lipid from freeze-dried lipovitellin by solvents*

Lipid in the lipoprotein (%)	Solvent	Temp. (°)	No. of 30 min. extractions	Percentage of total lipid extracted
29.1	Ethanol-ether, 1:1 (v/v)	-7	5	76.8
29.1	Ethanol	-28	5*	77.4
25.7	Ethanol	-7	4*	88.3
25.7	Ethanol	+25	4*	97.9

* Followed by two extractions with ether.

followed by evaporation of the solvent in a rotary film vacuum evaporator at $<40^{\circ}$. In each case ethanol was thoroughly removed from the protein before the temperature was allowed to rise, by drawing cold air through the protein on a sintered-glass filter for 1 hr., followed by two 30 min. extractions with ether and further aeration for 1 hr. Hewitt (1927) has pointed out that such thorough removal of ethanol is necessary if the denaturation of plasma proteins is to be prevented. Since extraction of the lipid was far from complete at -28° (Table 2), whilst at 25° the protein would certainly be damaged, extraction with ethanol at -7° (followed by ether as described above) was adopted as a compromise.

Emulsification of the lipid and of the lipid-protein mixture. Notwithstanding the known properties of phospholipins as emulsifying agents for oil-water systems, the dispersal of the free lipid in water for oxidation studies proved to be a matter of some difficulty. Two small impulse-type homogenizers tested had working parts of plated copper alloy and gave excellent emulsions, but enormously accelerated oxidation owing to metallic contamination. Replacement of the piston and cylinder by stainless steel resulted in rapid abrasion and consequent contamination. The all-glass apparatus finally employed was constructed from the lower half of a Pyrex glass boiling tube fused to a glass rod and rotated inside a slightly larger glass tube by a motor with a flexible coupling. After 'running in' with a little emery paste the fit of the tubes was such that the outer dropped only very slowly (or not at all) from the inner under its own weight. In operation the outer tube was slowly raised and lowered, thus pumping the fluid through the gap between rotor and stator. The apparatus was used in a room at $0-3^{\circ}$ to minimize heating effects. Emulsions obtained in this way were neither so fine nor so uniform as those obtainable with a metal homogenizer or colloid mill, but were uncontaminated and oxidized at reasonably reproducible rates.

Free lipid in the lipid-protein mixture. When the lipid and protein constituents of lipovitellin, separated by extraction of the freeze-dried lipoprotein with ethanol and ether at -7° or at $+25^{\circ}$, were re-emulsified together in water and the free lipid content of the mixture determined, it was found that not all of the added lipid could be re-extracted with ether. Thus, from a specimen of lipovitellin containing 25.7% of total lipid, 22.7% lipid was separated by repeated extraction with ethanol and ether at -7° . After re-emulsification of this lipid with the protein, however, only 20.6% lipid could be recovered by prolonged extraction with ether. In a second experiment 25.2% lipid was separated by extraction with ethanol and ether at 25° , but after re-emulsification with the protein only 22.1% could be recovered by ether extraction.

While these observations do indicate a certain degree of holding or binding of the lipid by the protein in the presence of water, they do not constitute proof of the regeneration of any part of the original lipovitellin complex.

Measurement of absorption of oxygen

The stock lipovitellin suspension was diluted to contain 100 mg. lipid/3.5 g. and this quantity, as delivered by a calibrated all-glass syringe, was placed in a Warburg flask together with (usually) 0.5 ml. 0.1M-phosphate or 0.25M-acetate, with 0.3 ml. catalyst solution in the side bulb and water to a total of 5 ml. in the flask. The buffers had previously been adjusted by titration with the glass electrode in

presence of the required concentration of lipoprotein and salt to give a final pH of 6.8 or 5.2 respectively. 0.8% NaCl was usually added, to bring the total ionic strength into the physiological region. KOH was used in the centre cups, and an atmosphere of O_2 in the flasks. The top of the bath was partly covered, and the water dyed dark red to prevent acceleration of the oxidation by the ultraviolet and blue components of daylight.

Since runs were usually continued for 30 hr. at 40° , precautions had to be taken against microbial growth, despite the somewhat unfavourable nature of the medium and low initial counts. Increasing the temperature to 45° reduced bacterial growth, but increased decomposition of the lipoprotein. Experiments were therefore carried out in duplicate at 40° with replacement of the NaCl by NaF. Typical 37° counts/ml. after 48 hr. at 40° were $>10^6$ with chloride, <10 with fluoride at pH 6.8, and 20-30 with chloride, <10 with fluoride at pH 5.2. Decomposition of the lipovitellin during 30 hr. as measured by the liberation of free lipid was of the order of 4% of the total lipid at pH 6.8 and 8% at pH 5.2. The effects of the buffer salts and of chloride and fluoride on the rate of oxidation were investigated separately.

Catalysts. Since tissues in which lipoproteins or phospholipins are present frequently also contain known catalysts for the oxidation of unsaturated fatty acids, comparisons of the rate of oxidation of lipovitellin with that of its lipid constituent were also carried out in the presence of haemoglobin, of cytochrome c and of haemin. The effect of ionic copper, which is liable to be present in food or in biochemical preparations as a contaminant, was also investigated.

Haemoglobin. Oxyhaemoglobin was prepared as required from a concentrated solution of purified ox haemoglobin preserved as the CO compound at -20° and diluted to contain 5 μ g. iron/0.3 ml.

Cytochrome c. Stock solutions of horse-heart preparations (Keilin & Hartree, 1945) were diluted to contain 5 μ g. iron/0.3 ml.

Haemin. Haemin (ferrihaem chloride) was dissolved in dilute NaOH and diluted to contain 5 μ g. iron/0.3 ml. Acid equivalent to the alkali used was added to the Warburg flask.

Copper. A slightly acidified $CuSO_4$ solution was diluted to contain 5 or 1 μ g. Cu/0.3 ml. Alkali equivalent to the acid used was added to the Warburg flask.

RESULTS

Effect of pH and of phosphate, acetate, chloride and fluoride on the rate of oxidation

Lipovitellin. Uncatalysed lipovitellin at pH 6.8 or 5.2 absorbed oxygen at an approximately linear rate over long periods. Neither phosphate nor acetate at the concentrations used had any appreciable effect on the rate of oxidation (Table 3). Sodium chloride (0.8%, w/v) nearly doubled the rate of oxidation at pH 5.2, although at pH 6.8 it was without effect. Fluoride had no effect at either pH. Decreasing the pH from 6.8 to 5.2 increased the rate of oxidation by 50-60% in the absence of added chloride, and approximately trebled the rate in its presence.

Table 3. *Effect of pH, chloride, fluoride, phosphate and acetate on the rate of oxidation* of lipovitellin*

Buffer	Salt (%, w/v)	Lipovitellin preparation no.				Average	Ratio:
		6	10	11	12		pH 5.2
							pH 6.8
—†	—	1.1	0.9	1.1	1.2	1.1	—
0.01 M-Phosphate	—	1.0	1.0	1.0	1.0	1.0	—
0.01 M-Phosphate	NaCl, 0.8	0.8	0.7	0.8	0.9	0.8	—
0.01 M-Phosphate	NaF, 0.8	1.1	0.8	0.8	1.0	0.9	—
		pH 5.2					
—†	—	1.3	2.0	1.7	2.3	1.8	1.6
0.025 M-Acetate	—	1.4	1.4	1.6	1.7	1.5	1.5
0.025 M-Acetate	NaCl, 0.8	2.3	2.8	2.3	3.3	2.7	3.4
0.025 M-Acetate	NaF, 0.8	1.2	1.4	1.4	1.8	1.5	1.6

* Average rate of oxidation, period 0–30 hr., based on rate in phosphate buffer without salt as 1.0 (actual average rate under these conditions = 6.9 μ l. O₂/100 mg. lipid/hr.).

† Self buffered; little or no pH change occurred.

Table 4. *Effect of pH, chloride, fluoride, phosphate and acetate on the rate of oxidation* of 'split' lipovitellin*

Buffer	Salt (%, w/v)	Lipovitellin preparation no.			Average	Ratio:
		10	11	12		pH 5.2
						pH 6.8
pH 6.8						
—†	—	1.0	1.0	1.0	1.0	—
0.01 M-Phosphate	—	1.0	1.0	1.0	1.0	—
0.01 M-Phosphate	NaCl, 0.8	0.8	0.8	0.9	0.8	—
0.01 M-Phosphate	NaF, 0.8	1.1	1.4	1.3	1.3	—
pH 5.2						
—†	—	1.1	1.1	1.0	1.1	1.1
0.025 M-Acetate	—	0.9	1.4	1.5	1.3	1.3
0.025 M-Acetate	NaCl, 0.8	1.1	1.8	1.6	1.5	1.9
0.025 M-Acetate	NaF, 0.8	1.1	1.8	1.7	1.5	1.2

* Average rate of oxidation, period 0–30 hr., based on rate in phosphate buffer without salt as 1.0 (actual average rate under these conditions = 3.3 μ l. O₂/100 mg. lipid/hr.).

† Self buffered; little or no pH change occurred.

Further investigation of the influence of sodium chloride on the oxidation of lipovitellin showed that at pH 5.2 the maximum acceleration appeared to be

Fig. 2. Effect of 0–6% sodium chloride and of 0.8% sodium fluoride on the oxidation of lipovitellin in buffer at pH 5.2 and 6.8.

reached at a concentration in the neighbourhood of 0.8%, whilst higher concentrations of chloride produced a gradual decrease in rate. At pH 6.8 increasing concentrations of chloride produced a

gradual decrease in rate without the initial acceleration (Fig. 2). Oxidation of lipovitellin at pH 5.2 in the presence of 0.8% sodium chloride in (a) 100% oxygen, (b) 88% oxygen, 12% nitrogen, and (c) 75% oxygen, 25% nitrogen (the latter two atmospheres corresponding approximately to the reduced solubility of oxygen produced by increasing the salt concentration from 0.8 to 3 and 6% (w/v) respectively), however, indicated that the falling off in rate of oxidation with increasing concentration of sodium chloride was probably due to the decreasing solubility of oxygen under these conditions. The direct effect of sodium chloride at pH 5.2 therefore appears to reach a maximum at 1% or below, and thereafter to remain constant up to 6% at least. This salt, apparently, had little or no direct effect on the rate of oxidation at pH 6.8.

'Split' lipovitellin. Neither phosphate nor acetate, chloride nor fluoride had any marked effect on the rate of oxidation of the lipid-protein mixture produced by splitting off the lipid from lipovitellin and subsequently re-emulsifying it with the protein. Oxidation tended to be slightly faster at pH 5.2 than at pH 6.8, particularly in the presence of sodium chloride (Table 4).

Lipid. Oxidations of the emulsified free lipid were carried out only in the presence of buffer. Little, if any, effect of chloride or fluoride was apparent, and the influence of pH, over the limited range covered, appeared to be small.

Relative rates of oxidation of lipovitellin and of 'split' lipovitellin (uncatalysed reaction)

The rates of absorption of oxygen by four preparations of lipovitellin, and by the same preparations after splitting off the lipid with ethanol at -7° and re-emulsifying with the protein, as described above, are compared in Fig. 3. In every case

Fig. 3. Relative rates of oxidation of lipovitellin (—) and of 'split' lipovitellin (---) in buffered suspension in the presence of chloride or fluoride. Preparations 9–12.

the lipoprotein oxidized more rapidly than the split product. At pH 6.8 the ratio of rates was rather less than 2:1; at pH 5.2 the ratio was about 2.5:1 in the presence of fluoride and over 3:1 in the presence of chloride.

Influence of catalysts in the three systems

The effects of copper, haemin, haemoglobin and cytochrome *c* on the oxidation of lipovitellin, of 'split' lipovitellin and of the free lipid are shown in Figs. 4–7.

Copper. Copper greatly accelerated the oxidation of the free lipid at both pH values (Fig. 4). The presence of protein in the system, whether free or bound to the lipid, greatly reduced the accelerating action of the copper and, in the case of the 'split' lipovitellin, eliminated it almost completely.

Haemin. Haemin showed powerful catalytic activity in all three systems, acceleration being greatest with the lipid and least with the lipoprotein

Fig. 4. Effect of copper (1 μ g. and 5 μ g.) on the oxidation of lipovitellin (LV), 'split' lipovitellin (SLV) and the separated lipid (L) at pH 6.8 and 5.2 in buffered NaCl (—) and NaF (---). C = control.

Fig. 5. Effect of haemin (5 μ g. Fe) on the oxidation of lipovitellin (LV), 'split' lipovitellin (SLV) and the separated lipid (L) at pH 6.8 and 5.2 in buffered NaCl (—) and NaF (---). C = control.

(Fig. 5). The shape of the oxygen absorption curves in all cases indicated a probable destruction of the catalyst as oxidation proceeded. This behaviour is in accord with the observations of Haurowitz, Schwerin & Yenson (1941), who found a destruction of haemin or haemoglobin to be coupled with the

oxidation of linoleic or linolenic acid in air at 38°, a considerable proportion of the haematin iron being liberated ultimately in the inorganic state.

Haemoglobin. Haemoglobin showed about the same accelerating activity as haemin in lipovitellin, but, in contrast to haemin, was comparatively ineffective with the free lipid (Fig. 6). The curves again provided evidence of a destruction of the catalyst as oxidation proceeded.

Fig. 6. Effect of haemoglobin (5 µg. Fe) on the oxidation of lipovitellin (LV), 'split' lipovitellin (SLV) and the separated lipid (L) at pH 6.8 and 5.2 in buffered NaCl (—) and NaF (---). C = control.

Cytochrome c. The effect of cytochrome c on oxidation was small and the results have not been presented in detail. With lipovitellin a slight but definite and consistent retardation was observed. With 'split' lipovitellin the effect of cytochrome c was negligible. With the free lipid oxidation was slightly accelerated by cytochrome c in the presence of chloride, particularly at the more acid pH, but the effect was small and appeared to be inhibited by fluoride. Experiments with other specimens of cytochrome at concentrations of 0.5–5 µg. iron gave similar results.

DISCUSSION

A major difficulty, which prevented a rigid comparison of the rates of oxidation of the lipoprotein complex, of the lipid-protein mixture ('split' lipoprotein) and of the isolated lipid component itself, was the impossibility of obtaining the lipid in the three systems in the same physical state, particularly with respect to degree of dispersion. Despite this handicap, the results clearly afford no support for the widely held view that lipids of lipoproteins are greatly stabilized against oxidation by oxygen. Thus, although dried plasma, if stored in the presence of oxygen, develops rancidity quite

readily (Ratcliff, 1950), it has been pointed out that there is no evidence of rapid oxidation of lecithin in normal plasma (Frazer, 1949). Even with particular reference to lipovitellin, Fevold & Lausten (1946) write: 'Another fact that has become apparent in this work is the increased stability of the phospholipins when in combination with the proteins. The combination appears to be a somewhat loose one as judged by the ease of breaking. However, the typical browning of uncombined phospholipins on exposure to air takes place only very slowly in the phospholipins combined in the lipoproteins.' In the present work, when the lipid and protein fractions of lipovitellin were separated with some care, remixed, and subjected to the same carefully controlled oxidizing conditions as the original lipoprotein, no increase in rate of oxidation was observed. On the contrary, an appreciable decrease in the rate of oxidation occurred, although no particular significance has been attached to it in view of experimental limitations and possible partial denaturation of protein during separation. In certain complex food systems, e.g. milk (Findlay, Higginbottom, Smith & Lea, 1946), heating under conditions which expose protein sulphhydryl groups has been found to increase the resistance of the fat present to autoxidation. Free phosphatides are normally preserved in the dry state and lipoproteins in an aqueous phase, which reduces the concentration of oxygen in contact with the lipid by about forty times for normal saline or sixty times for the 10% sodium chloride used by Fevold & Lausten to hold lipovitellin in solution (values calculated for 30°). This may account for part at least of the observed behaviour. Lipoprotein may be completely protected from autoxidation inside tissues such as muscle, which possess an appreciable residual oxygen uptake.

Since the same general result was obtained in the presence of chloride or fluoride, the probable absence of significant interference by bacterial growth in the chloride experiments or of specific interference by fluoride could be inferred. It was observed, however, that whilst the oxidation of the 'split' mixture was not appreciably influenced by pH (6.8 or 5.2) or by the nature of the salt present (sodium chloride or fluoride), the intact lipoprotein oxidized appreciably more rapidly at the more acid pH and, at this pH, was more readily oxidizable in the presence of chloride than of fluoride (Fig. 3). The observed effect of chloride (Fig. 2) may have been somewhat too low, since no particular precautions were taken to free the lipovitellin from the last traces of the chloride used so freely in its preparation. The mechanism of the action of chloride is unlikely to be connected with the solubility of the lipoprotein, because the maximum accelerating effect was reached at salt concentrations too low seriously to affect solubility. Since activity at pH 5.2 but not at

pH 6.8, and activation by chloride but probably not by fluoride, are properties of the ill defined unsaturated fatty acid 'lipoxidase' of mammalian muscle (Lea, 1937), it is possible that contamination with such an enzyme rather than the nature of the lipoprotein itself might be responsible for the observed results. In the absence of adequate data on the properties of the 'lipoxidase', it was not considered profitable to pursue the matter further at this stage.

Concerning the effects of catalysts, combination with protein undoubtedly largely protected the lipid from the catalytic action of copper, presumably owing to the binding of a large proportion of the copper in a non-ionic form. But combination with protein was no better in this respect than simple admixture; in fact copper was inactivated even more efficiently by the free than by the combined protein (Fig. 4). This might have been due either (a) to copper being bound by protein groups which in turn are combined with lipid in the lipoprotein, or (b) to a certain degree of denaturation of the protein, with unmasking of copper-binding sulphhydryl groups, during removal of the lipid from the lipoprotein.

In catalysis by haemin, the presence of protein afforded partial although not complete protection, and in this case combination with the protein was appreciably more effective than simple admixture (Fig. 5). Almost the reverse was true with haemoglobin where reactive groups of the free phosphatide appear to have inactivated the catalyst more readily in the absence of other protein.

Cytochrome *c*, found by Banks (1949) to be the most active of the natural haematin pigments for the oxidation of linoleic acid emulsions in phosphate at pH 6.6, was comparatively ineffective as a catalyst for the oxidation of the lipid (mainly phospholipin) of lipovitellin under all conditions tested.

The action of ethanol in disrupting the lipid-protein linkage is widely known and utilized in extraction procedures, although the mechanism of its action (often assumed to be one of dehydration) and even the nature of the union between lipid and protein is largely unknown. Some of the lipid, particularly the more inert fractions such as

cholesterol and its esters, appears to exist as solid solution in the prosthetic portion of the lipoprotein. For this reason the action of limited quantities of ethanol at -7° to $+25^{\circ}$ in rendering only 40–50% of the lipid extractable with ether may be of value as a possible means of fractionating the bound lipid. In this connexion Delsal (1949) has recently observed that extraction of blood serum with ether at -70° according to the procedure of McFarlane (1942) removes only cholesterol from serum, leaving the phospholipin-protein complex intact. The observation that a fraction of the lipid, although only a comparatively small one, becomes unextractable with ether when the separated lipid and protein components of lipovitellin are remixed is also of some interest.

SUMMARY

1. Lipovitellin from egg yolk has been split by treatment with ethanol at low temperature into its lipid and protein components and the rates of autoxidation at 37° of the original lipoprotein, of the remixed lipid and protein, and of the lipid alone compared at pH 6.8 and 5.2.

2. In the uncatalysed systems no support could be found for the widely held view that phospholipins are greatly stabilized against autoxidation by combination with protein.

3. Combination with protein gave considerable protection against the catalytic action of copper and of haemin, but not against the (weaker) action of haemoglobin. Copper, however, was at least as effectively inactivated by free protein as when the latter was combined with the lipid.

4. Cytochrome *c* had very little catalytic action on the oxidation of lipid of lipovitellin whether free or combined.

5. The lipovitellin preparations used autoxidized more rapidly at pH 5.2 than at pH 6.8, and at the more acid pH the rate was further accelerated by the presence of chloride but not of fluoride.

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