The Lipoprotein Particles in Cow's Milk

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Normal cow's milk has long been recognized to contain at least three types of particles, namely: 'foreign' material, such as udder cells, leucocytes and bacteria; fat globules, which vary considerably in size; and colloidal particles of the casein complex. Although the physical and chemical properties of casein have been the subject of much investigation (see Sutermeister & Browne, 1939; McMeekin & Polis, 1949) there have been no previous studies of the relationship of the particulate protein complexes to the enzymic activities of milk. Preliminary studies on the purification of alkaline phosphatase of milk, however, indicated that this enzyme did not occur in true solution but was entirely associated with a particulate component of a lipoprotein nature, which had not previously been recognized in milk (Morton, 1953*a*).

In order to characterize this component further it has been separated from the more abundant casein complex using a procedure expected to produce only a minimum of denaturation or other modification. The procedure is described in this paper. The chemical and enzymic properties of the lipoprotein particles have been compared with those of casein particles isolated at the same time and both complexes have been examined using the electron microscope. Some aspects of this work have been reported (Morton, 1953*b*).

MATERIALS AND METHODS

Enzyme substrates

β-Glycerophosphate (sodium salt) was a commercial preparation (British Drug Houses Ltd.).

Hypoxanthine was a pure preparation, kindly supplied by Dr M. Dixon, F.R.S.

Diphosphopyridine nucleotide (*DPN*) of 30% purity, as determined by reduction using crystalline alcohol dehydrogenase, was kindly supplied by Mr E. J. Morgan. It was reduced enzymically using crystalline alcohol dehydrogenase prepared from yeast according to Racker (1950).

Cytochrome c was prepared from horse heart by Mr E. J. Morgan, using the procedure of Keilin & Hartree (1945).

Solvents

Solvents were purified by distillation, either over NaOH (butanol) or over sodium (diethyl ether).

Determination of enzymic activities

Alkaline phosphatase. This was estimated from the rate of hydrolysis of β-glycerophosphate (sodium salt) essentially as described previously (Morton, 1953*a*).

Dehydrogenases. Activities were determined by measuring the rate of reduction of 2:6-dichlorophenol indophenol at 600 mμ., using a Beckman Spectrophotometer (model DU). Measurements were made in 1 cm. cuvettes at room temp. (approx. 18°) in potassium phosphate buffer (pH 7·3; final concentration 0·075 M). The final volume in each cuvette was 3·0 ml. and the final concentration of dye was 6×10^{-5} M. The concentrations of substrates for the various activities were: for xanthine oxidase, hypoxanthine, 0·0033 M; for diaphorase, reduced DPN, $1{\cdot}35 \times 10^{-4}$ M; for succinic dehydrogenase, sodium succinate, 0·065 M.

DPN-cytochrome c *reductase.* The rate of reduction of cytochrome c at 550 mμ. was measured essentially as described by Slater (1950). Final concentrations of reactants were: potassium phosphate buffer (pH 7·3), 0·075 M; reduced DPN, $1{\cdot}35 \times 10^{-4}$ M; oxidized cytochrome c, 5×10^{-5} M.

Chemical methods

Dry weight was determined after drying from the frozen state over P_2O_5 for 48 hr. *in vacuo* at room temp. (approx. 18°).

Nitrogen was determined by a colorimetric Kjeldahl procedure using Nessler reagent (Johnson, 1941).

Total lipid and phospholipid. A portion (10–50 mg.) of the dried material was suspended in 5 ml. of butanol, held 15 min. at room temp. (approx. 18°) and centrifuged. The supernatant butanol was carefully removed. The procedure was repeated twice, and the precipitate washed twice with two 5 ml. portions of diethyl ether. Total lipid was estimated by the loss in dry weight. The combined solvents (butanol and ether) were removed by vacuum distillation (at 60–65°), the residue dissolved in ether, and a suitable portion used for determination of phosphorus by the method of Fiske & Subbarow (1925).

Nucleic acid. 'Acid-soluble phosphates' were removed by extracting the lipid-free material with 0·5 N-$HClO_4$ at 0° for 4 hr. Nucleic acids were then removed by two extractions with 5 ml. N-$HClO_4$ at 80° for 15 min. The combined extracts were adjusted to pH 7·2 with 5 N-KOH; the insoluble potassium perchlorate was removed by centrifuging, the precipitate washed once and the supernatant and washings were combined. The absorption curve at wavelengths between 255 and 270 mμ. was determined using a Beckman Spectrophotometer with a 1 cm. light path. The maximum reading (at 262 mμ.) was converted to nucleic acid P using a molecular extinction coefficient (based on ribonucleic acid P) of 10800 (Ogur & Rosen, 1950). It was assumed that the absorption at 262 mμ. was entirely due to ribonucleic acid.

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EXPERIMENTS AND RESULTS

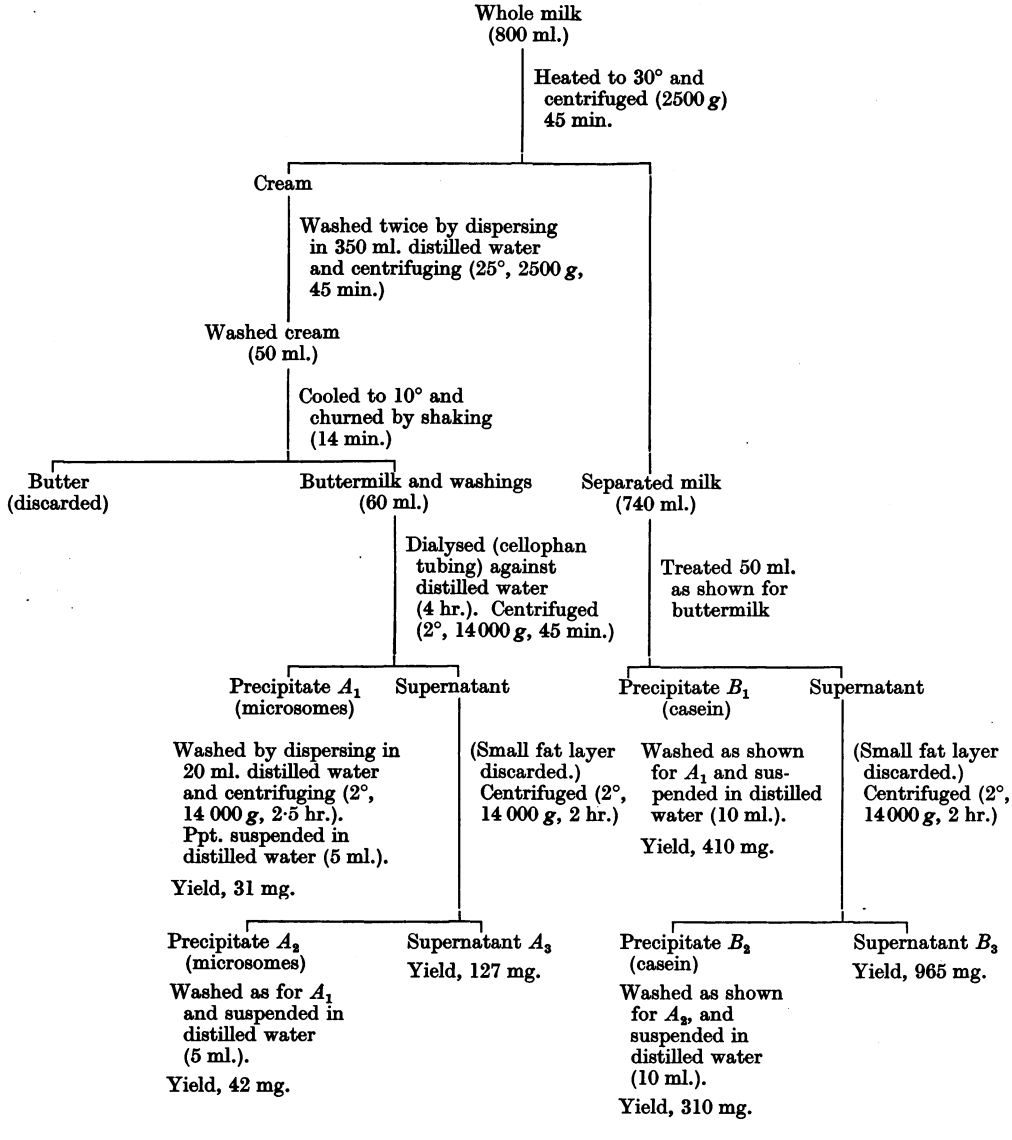
Preparation of the particulate components of normal whole milk

Preliminary studies had indicated the considerable difficulty in separating the lipoprotein particles carrying the alkaline phosphatase activity from the casein particles (Morton, 1953*a*). In order to separate the two types of particles with a minimum of denaturation, advantage was taken of the tendency of the milk-fat globules to adsorb lipoprotein particles.

Milk was obtained in February 1952 from a commercial herd of about twenty Jersey cows, most of which were nearing completion of their lactation periods. The sample was drawn from the tank of mixed morning milk within 2 hr. of commencement of milking, cooled to about 15° and transported to the laboratory immediately.

The details of the further treatment are shown in the diagram below.

By this procedure both separated milk and buttermilk were fractionated into two precipitates and a final supernatant, discarding a fatty layer on each occasion. Portions of each fraction were removed for enzymic tests and electron micrograph studies, and the remainder was dried from the frozen state over P_2O_5 *in vacuo* at room temperature (approx. 18°) and used for chemical analyses.



Comparative study of properties of the particulate components

A comparison of the properties of the two precipitates from the different milk fractions (buttermilk and separated milk) clearly indicates the difference between the two types of particles.

When well-packed, the precipitates (B_1 and B_2) from the separated milk formed a clear, translucent pellet, faintly blue in colour. They readily dispersed in water to form stable, opaque-white suspensions. In contrast, both precipitates from buttermilk (A_1 and A_2) were opaque and orange-pink in colour. In distilled water they formed stable, brown suspensions.

A thick smear of each of the various precipitates was examined with a microspectroscope. Quite strong bands were seen at approx. 552 and 525 mμ. in both the precipitates A_1 and A_2, but no bands could be seen in either of the other precipitates, even when they were reduced with $Na_2S_2O_4$. There was no discernible increase in intensity of the bands of the precipitates A_1 and A_2 when $Na_2S_2O_4$ was added. These bands are attributed to the presence of cytochrome *c* in precipitates A_1 and A_2, although there is a slight discrepancy between the observed wavelengths and those of the α- and β-bands of reduced cytochrome *c* (550 and 521 mμ.).

Chemical analyses. The yields of each of the fractions obtained from the milk are shown in the diagram of the separation procedure. The results of the chemical analyses are given in Table 1. It may be seen that the particles from the buttermilk contained relatively large amounts of lipid, protein and nucleic acid, and are therefore referred to as lipoprotein particles in the following sections.

Enzymic properties. The alkaline phosphatase, diaphorase, DPN-cytochrome *c* reductase, xanthine oxidase (determined by dehydrogenase activity) and succinic dehydrogenase activities were estimated for each fraction.

Although all fractions were turbid, it was found that consistent results could be obtained by measurement of dehydrogenase and reductase activities spectrophotometrically, since the suspensions were quite stable. Readings were made after thorough mixing of the contents of the cuvettes by using thin polythene rods to avoid damage to the optical faces. The reactions were started by the addition of substrates. Substrates were omitted from the blank cuvettes.

Since the lipoprotein particles possessed DPN-cytochrome *c* reductase activity but no succinic dehydrogenase activity, it was of interest to determine whether cytochrome oxidase was present. This seemed unlikely since the cytochrome *c* in the freshly isolated particles appeared to be fully reduced. Addition of potassium cyanide (final concentration 0·001 M) to the cuvettes caused no change in the rate of reduction of added cytochrome *c* by the buttermilk fractions (A_1 and A_2). Furthermore, qualitative tests for cytochrome oxidase using Nadi reagent with catalytic amounts of added cytochrome *c* were completely negative. It was therefore concluded that no cytochrome oxidase activity was present in any of the precipitates.

The enzymic activities of the various fractions are shown in Table 2.

Table 1. *Chemical analyses on milk fractions*

	% dry wt.					
	Fractions from buttermilk			Fractions from separated milk		
	A_1	A_2	A_3	B_1	B_2	B_3
Total lipid	22·1	16·8	—	3·5	5·7	—
Lipid P	0·77	0·62	—	0·11	0·26	—
Total N	9·7	11·2	14·2	13·7	11·9	15·8
Nucleic acid P	1·18	0·95	—	—	—	—

Table 2. *Enzymic activities of milk fractions*

	Units of activity as specified for each enzyme					
	Fractions from buttermilk			Fractions from separated milk		
	A_1	A_2	A_3	B_1	B_2	B_3
Alkaline phosphatase*	57·2	36·8	8·7	0·6	3·1	1·2
Xanthine oxidase†	37·7	23·4	5·3	0	0·2	0
Diaphorase†	2·5	2·4	1·2	0	0·1	0
DPN-cytochrome *c* reductase‡	0·1	0·1	0	0	0	0
Succinic dehydrogenase†	0	0	0	0	0	0

* μmoles P liberated/hr./mg. N from β-glycerophosphate (at 37°).
† μmoles 2:6-dichlorophenol indophenol reduced/hr./mg. N (at 18°).
‡ μmoles of cytochrome *c* reduced/hr./mg. N (at 18°).

Size and structure. A number of dilutions in distilled water from each of the precipitates (A_1, A_2 and B_1, B_2) and supernatants (A_3 and B_3) were prepared and used directly for dried preparations on nitrocellulose supporting films. The preparations were dried *in vacuo*, shadowed with gold-palladium (angle approx. 20°), and then examined in a Siemens electron microscope, using a high-tension voltage of 70 kv.

Pl. 1, fig. 1, shows an electron micrograph of a typical field of the particles from separated milk (precipitate B_1). Such casein particles frequently exhibited packing in regular clumps, and this appeared to be a distinctive feature.

A typical field of lipoprotein particles obtained from the buttermilk (precipitate A_1) is shown in Pl. 1, fig. 2. On comparison with the casein particles (Pl. 1, fig. 1), it is seen that the lipoprotein particles appear somewhat flattened, more variable in size and shape, and much less dense. They frequently showed aggregation into indefinite clumps.

It will be seen from Pl. 1, figs. 1 and 2, that many of the particles of both the casein and lipoprotein complexes are of similar size range (approx. 200 mμ. to about 30 mμ.). Both types, however, were observed to have a distribution in size extending beyond the limits of resolution with the equipment available (less than 30 mμ. diameter).

Pl. 1, fig. 3, shows an electron micrograph of the supernatant (A_3) remaining after centrifuging buttermilk. Apparently some of the smaller fat globules still remained in this supernatant. In the upper centre portion of the plate is seen an intact fat globule, of about 700 mμ. diameter, with a protein membrane on to which lipoprotein particles are apparently adsorbed. A 'crater' which has been formed by collapse of a fat globule is seen particularly well towards the left-hand edge of this plate.

DISCUSSION

In order to characterize further the lipoprotein complex which was identified in milk during purification of the alkaline phosphatase (Morton, 1953*a*), it was considered desirable to isolate the complex by using methods expected to cause only a minimum of modification. This was achieved by: (1) concentration of the lipoprotein particles in the cream layer; (2) selective removal of casein by washing of the fat globules, which adsorb the lipoprotein particles more strongly than casein (cf. Hansson, Solberg & Sjöström, 1946); (3) release of the lipoprotein particles into the serum (buttermilk) on phase-inversion from cream to butter; (4) sedimentation by high-speed centrifuging.

The same procedure gave separated milk enriched in casein and depleted of lipoprotein. High-speed centrifuging permitted some further fractionation of the two types of particles, since some of the larger casein particles are sedimented from separated milk before the lipoprotein particles (see Morton, 1953*a*).

In this way two types of precipitates were obtained from whole milk—precipitates A_1 and A_2 from buttermilk, and B_1 and B_2 from the residual, separated milk. The differences between the two types of precipitates in appearance and colour, and in chemical, physical and enzymic properties establishes that normal cow's milk contains at least two different readily sedimentable, protein-containing complexes. The properties of the precipitates from the separated milk (B_1 and B_2) correspond closely with those of the well-known casein complex; the properties of the precipitates from the buttermilk (A_1 and A_2) with those of the lipoprotein complex previously obtained by chemical fractionation of milk (Morton, 1953*a*).

The casein complex

The electron micrograph (Pl. 1, fig. 1) shows that the casein complex (which represents about 85 % of the total protein of cow's milk) occurs as discrete particles, which are almost certainly spherical. Some flattening probably occurs during the drying of the specimens. The field is representative only of those particles which could be sedimented from the separated milk at the centrifugal force available (14 000 *g*) and therefore shows only the larger casein particles. Furthermore, within the fields examined there were many particles which could not be properly resolved with the equipment available. By inspection it appears that the size range of the casein particles extends from a maximum of 200 mμ. diameter to less than 30 mμ. This is in agreement with earlier estimates by a number of workers based on other physical methods (see Eilers, Saal & van der Waarden, 1947), and with the direct measurements by Nitschmann (1949) from electron micrographs of diluted milks. There appears to be a limited number of particle sizes (cf. Ford & Ramsdell, 1949) and the majority probably fall in the size range 67–80 mμ., as found by Nitschmann (1949).

Recently, Heyndrickx & de Vleeschauwer (1952), by electrophoresis of sera obtained by centrifuging milk, recognized three protein components (designated as *B*, *C* and *G*) with the sedimentation characteristics of casein. The *B* and *G* components were considered by these workers to be identical with α- and β-casein, respectively, but the nature of the *C* component was not discussed.

These workers found that the *C* component comprised 7·9 % of the total protein of normal milk, but that there was more in colostrum whereas there was less of components *B* and *G*. On centrifuging, component *C* sedimented more slowly than either

B or *G*. These characteristics are very similar to those of the enzymically active lipoprotein complex. This complex comprises about 5 % of the total milk protein by weight (see below). The alkaline phosphatase (and hence the lipoprotein) is higher in colostrum than in normal milk (Folley & Kay, 1936). Moreover, the lipoprotein settles from separated milk more slowly than casein (Morton, 1953*a*). Hence it seems quite possible that component *C* of Heyndrickx & de Vleeschauwer is not a casein protein but the lipoprotein complex which, as shown below, consists of microsomal particles.

The lipoprotein complex

The analyses in Tables 1 and 2 show the differences between the two particulate protein complexes in milk. Comparison of the electron micrographs (Pl. 1, figs. 1 and 2) also reveals differences, although the particles of the two complexes are of a similar size range (maximum 200 mμ. to less than 30 mμ.).

Submicroscopic lipoprotein particles obtained from dispersions of animal tissues have been called 'microsomes'. The microsomes from mouse liver (Chantrenne, 1947; Barnum & Huseby, 1948) and mouse mammary gland (Barnum & Huseby, 1950) have a high phospholipid and nucleic acid content. Liver microsomes have an active DPN-cytochrome *c* reductase (Hogeboom, 1949) but lack succinic dehydrogenase and cytochrome oxidase (Hogeboom, Schneider & Pallade, 1948). According to conditions used for isolation, they may contain cytochrome *c* (see Schneider & Hogeboom, 1951). Moreover, the cytoplasmic alkaline phosphatase of rabbit (and guinea pig) kidney and intestine (Hers, Berthet, Berthet & de Duve, 1951) and of calf intestinal mucosa and cow mammary gland (Morton, 1954) is almost completely associated with the microsome fraction. Since the milk lipoprotein particles show essentially similar chemical and enzymic properties to the cytoplasmic microsomes, they will be called 'milk microsomes'.

Since the alkaline phosphatase of milk is almost completely associated with the lipoprotein particles (Morton, 1953*a*), the distribution of this enzyme indicates the distribution of the microsomes among milk fractions. About 20 % of milk alkaline phosphatase is recovered in buttermilk, but a loss of about 50 % would occur in washing the cream to remove casein particles (see Morton, 1953*a*). If the recovery of microsomes from the buttermilk in fractions A_1 and A_2 (total, 73 mg.) is assumed to be about 50 %, then these lipoprotein particles would appear to comprise about 5 % by weight of the total protein of the milk.

The nature of the red pigment of the milk microsomes has not been established. Although liver microsomes are a similar orange-pink colour (Chantrenne, 1947) the main pigment appears to be a haemochromogen which shows bands at 557 and 527 mμ. when in the reduced state (Strittmatter & Ball, 1952). No haemochromogen other than cytochrome *c* was observed in the milk microsomes but the concentration of this cytochrome was probably too low to account for the colour of the particles. It seems probable that the red pigment of milk microsomes is related to the red protein obtained during purification of alkaline phosphatase from buttermilk (Morton, 1950, 1953*c*).

Xanthine oxidase activity of milk microsomes

Xanthine oxidase has not previously been demonstrated as associated with microsomal fractions and therefore the considerable activity of the milk microsomes is noteworthy. While the quantitative distribution of this flavoprotein has not been studied, it is most unlikely that the high activity of the microsomes was due to adsorption of the enzyme from true solution, or indeed, that any xanthine oxidase occurs in true solution in milk. The casein particles, which would present a very much greater adsorbing surface, contained virtually no xanthine oxidase. Dixon & Thurlow (1924) observed that the enzyme could be precipitated with the casein fraction and partly extracted from this after treatment of the dried protein with diethyl ether to remove lipid material. They also showed that the enzyme could be adsorbed from milk on to a variety of materials, but difficulty was experienced in eluting the adsorbed enzyme, a finding confirmed by Dixon & Kodama (1926). This behaviour corresponds closely with that found for the particle-bound alkaline phosphatase (Morton, 1953*a*). Moreover, milk xanthine oxidase may be concentrated in the cream fraction and brought into solution by digestion with pancreatic lipase or crude trypsin (Ball, 1939; Horecker & Heppel, 1949) similarly to alkaline phosphatase (Zittle & Della Monica, 1950, 1952; Morton, 1953*a*). Furthermore, it may be seen from inspection of Table 2 that the ratio of the xanthine oxidase activities for fractions A_1, A_2 and A_3 (7:4:1) is similar to that of the alkaline phosphatase activities (7:4:1). It may be concluded, therefore, that milk xanthine oxidase is wholly bound to the microsomes and it is quite possible that both xanthine oxidase and alkaline phosphatase are attached to the one particle.

It is possible to calculate the relative amounts of xanthine oxidase and alkaline phosphatase protein associated with milk microsomes. Ball (1939) states that whole milk contains about 160 mg. xanthine oxidase/l. This figure should be adjusted to about 60 mg./l., allowing for the much higher activity of the enzyme as obtained by Horecker & Heppel (1949). Pure milk phosphatase has a specific activity of 15300 units (μg. P liberated/min.)/mg. N (Morton, 1953*c*) and whole milk contains about

14 units/ml., so that normal milk has $\frac{14 \times 10^3}{15300} \times 6{\cdot}25$ or approx. 6 mg. alkaline phosphatase protein/l. Hence there is about ten times more xanthine oxidase protein than alkaline phosphatase (by weight) in milk and if both enzymes are associated with the same microsomes, this would also be the relative amounts of the two proteins in these particles.

Origin of the milk microsomes

Folley & White (1936) and Folley & Kay (1936) have shown that milk alkaline phosphatase is derived from the mammary gland rather than from the blood serum, while the purified milk enzyme is now known to be the same as that from the mammary gland (Morton, 1952). Microsomes isolated from the mammary gland of the cow have similar enzymic activities to those obtained from the milk (Morton, 1954). Moreover, cytoplasmic enzymes such as catalase and peroxidase occur in true solution in milk, together with certain phosphorylated intermediates of cell metabolism (Graham & Kay, 1933; Chanda, McNaught & Owen, 1952; McGeown & Malpress, 1952). Hence it may be concluded that the microsomes of milk are derived directly from the secretory cells of the mammary gland.

The escape of some of the cytoplasmic components of the secretory cells is not surprising. The process of secretion involves (1) synthesis of the milk constituents in the functioning cells of the alveoli, and (2) ejection of the synthesized products into the alveolar lumen. It has not as yet been established whether the basal membrane of the secretory cells ruptures to release the milk and is reformed before the next secretory cycle, or whether the membrane is sufficiently permeable to offer little resistance to the passage of milk (Espe, 1946; Richardson, 1947). Whilst the release of microsomes might be considered as evidence in favour of the former mechanism, even if the latter hypothesis were correct the passage of particulate components such as casein across the cell membrane would almost certainly be accompanied by some loss of cytoplasmic material.

In view of this evidence that cytoplasmic components escape into the milk, it is particularly interesting that no succinic dehydrogenase or cytochrome oxidase was found in any of the milk fractions examined. This suggests that the mitochondria, with which these enzymes are so intimately associated, do not escape on ejection of the milk. This may be due to a spatial separation of the microsomes and mitochondria within the cytoplasm, or to a differential permeability of the upper membrane of the secretory cells. If the upper cell membrane is not ruptured, it may well be able to retain relatively large mitochondria while, at the same time, some of the microsomes escape together with the casein particles of similar size range. The ejection of the large fat globules, of course, probably involves a different mechanism from that applying to the particulate protein complexes.

Association of microsomes with the milk-fat membrane

A small fat globule which has fortuitously remained intact during drying of the preparation for examination in the electron microscope is seen in Pl. 1, fig. 3. Most of the fat globules, however, collapsed during the drying process and are seen as 'craters' with raised edges. It would appear from this photograph that the fat globule in milk is surrounded by a continuous protein membrane on to which microsomal particles may be adsorbed, since small particles can be seen adhering to both the intact and collapsed globules. Whether the microsomes are adsorbed on to the fat globules before or after the ejection of the globules from the secretory cells has not as yet been established, but it is quite possible that many of the microsomes are carried into the milk in association with the fat globules. The adsorbed microsomal lipoprotein could account for the phospholipid of the globule membrane which has been shown to exert a considerable influence on the behaviour of the milk-fat globule (Palmer & Wiese, 1933; Rimpila & Palmer, 1935; Moyer, 1940).

SUMMARY

1. The lipoprotein complex of cow's milk has been separated from the casein complex by using the marked tendency of the milk-fat globules to adsorb the lipoprotein. Electron micrographs show that both components occur in milk as discrete particles, diminishing in size from a maximum of about 200 mμ. to less than 30 mμ.

2. The brown lipoprotein particles were found to contain about 22% total lipid (largely phospholipid), nucleic acid, a haemochromogen (probably cytochrome *c*) and the following enzymic activities: alkaline phosphatase, xanthine oxidase, diaphorase, and DPN-cytochrome *c* reductase. Succinic dehydrogenase and cytochrome oxidase activities were absent.

3. The lipoprotein particles have properties essentially similar to those of microsomes from lactating mammary gland and other animal tissues and therefore are called 'milk microsomes'. They are released into milk from the mammary gland during the normal secretory process, and occur partly in association with the fat globule.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during the course of this work and Dr V. Cosslett for granting me facilities of the electron microscope at the Cavendish Laboratory, Cambridge. I am parti-

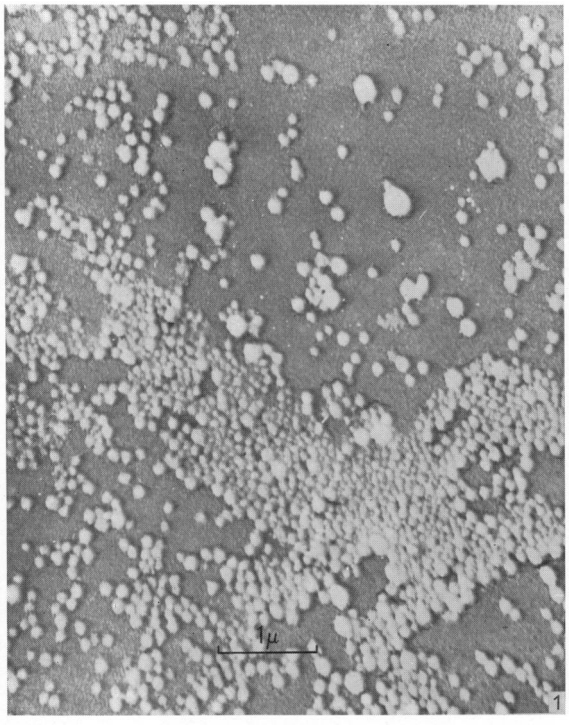


Fig. 1

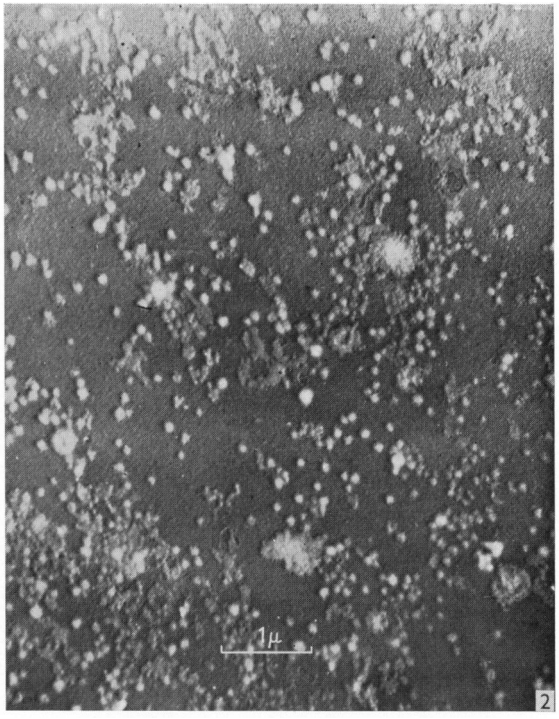


Fig. 2

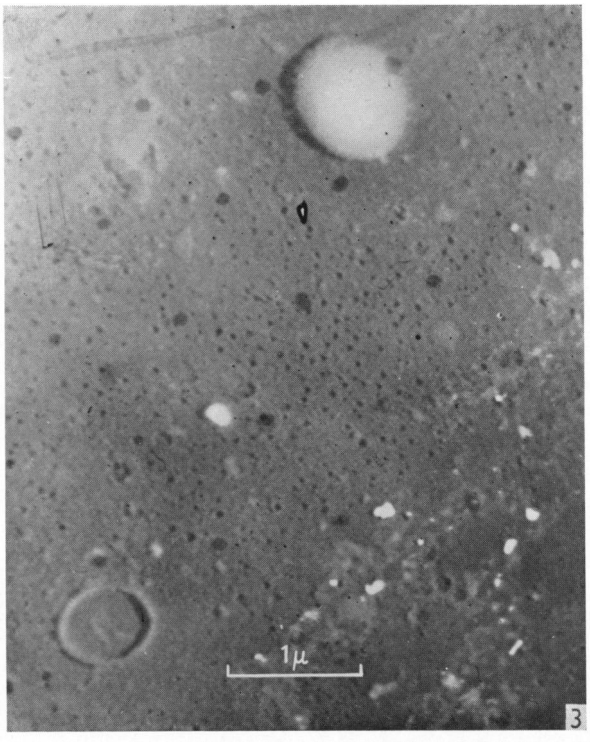


Fig. 3

cularly indebted to Mr R. Horne for taking the electron micrographs.

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EXPLANATION OF PLATE 1

Fig. 1. Electron micrograph of a representative field of casein particles (precipitate B_1) from separated milk fraction.

Fig. 2. Electron micrograph of a representative field of lipoprotein particles or 'milk microsomes' (precipitate A_1) from buttermilk fraction.

Fig. 3. Electron micrograph of a representative field of the final supernatant (A_3) from buttermilk fraction. Note an intact fat globule with a protein membrane (upper centre) and several collapsed fat globules.

Studies on the Labelling of Brain Phospholipids with Radioactive Phosphorus

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During the last decade extensive work with radioactive phosphorus (^{32}P) has shown that the phospholipids present in the adult mammalian brain are constantly being broken down and resynthesized. However, little attention has been paid to the relative rates at which the individual phospholipids are metabolized and become labelled with the isotope. This is largely due to the lack of suitable methods for the fractionation of brain phospholipids, especially when only small samples of tissue are available. The use of column and paper chromatography for this purpose has proved somewhat disappointing (Collins, private communication; Zechmeister, 1950; Lea & Rhodes, 1953), while

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