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COMPARATIVE STUDY OF THE MILK FAT GLOBULE MEMBRANE AND THE MOUSE MAMMARY TUMOUR VIRUS PREPARED FROM THE MILK OF AN INFECTED STRAIN OF SWISS ALBINO MICE

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SUMMARY

Milk fat globule membranes and mammary tumour virus particles ($d = 1.17$ g/cm^3) have been obtained from the milk of a Swiss albino mice strain. Comparative biochemistry shows that these two structures differ significantly in the phospholipid, polypeptide and glycopolypeptide patterns and enzymatic activities. However, the lipid profile and the morphology of both structures suggest a filiation with the plasma membrane. Density fractions obtained from the crude virus preparation have been thoroughly investigated. The results suggest that most of these fractions represent degraded virus and/or atypical virus assembly.

INTRODUCTION

The milk fat globules are formed in the cytoplasm of the mammary cell and excreted by a budding process involving most of the apical part of the plasma membrane [1–3]. These milk fat globules are surrounded by a membrane, the milk fat globule membrane, which is thought to derive from the mammary cell plasma membrane and which can be isolated by relatively simple and non-disruptive procedures [4].

Many strains of mice excrete large amounts of mammary tumour virus in their milk [5, 6]. This virus originates from those same cells that secrete the milk fat globules and matures by budding at the plasma membrane of the host cell [7, 8].

As the milk fat globule membrane and the mammary tumour virus envelope are presumed to share a common origin, isolation of each of these structures may provide an interesting model for the study of the complex interactions of virus and host. Accordingly, we purified milk fat globule membrane and mammary tumour virus from the milk of infected mice, in order to carry out a comparative study of the morphology and the chemical composition of these two types of membranous structures.

Preliminary accounts of this work have been presented [9, 10].

MATERIALS AND METHODS

Virus and milk fat globule membrane preparation

Mice. Our laboratory strain of Swiss albino mice, inbred for 20 years, was used, as it happened that large amounts of mammary tumour virus were excreted in their milk. 10–15 % of the multiparous females develop mammary carcinoma.

Milk. The mice were milked under anaesthesia (0.25 mg sodium pentobarbital intraperitoneally) with the aid of a small suction device designed by Leyten [11] after intraperitoneal injection of 0.2 I.U. oxytocin. The average milk yield was 1.2 ml per mouse. The milk, kept at room temperature until a volume of 20–25 ml was obtained, was then diluted with 3 vols. of a solution composed of 10 mM Tris · HCl (pH 7.2), 0.1 M NaCl and 30 mM EDTA, left at room temperature for another 30 min and centrifuged 10 min at 2800 rev./min to separate the cream from the skimmed milk. The cream was washed twice with 3 ml of the above solution; the washings were pooled with the skimmed milk. The skimmed milk was then centrifuged at $3000 \times g$ for 10 min at 20 °C (as all further centrifugations) to precipitate cells and debris.

Milk fat globule membranes. The washed cream (2–3 ml), cooled to 5 °C, was churned in a polyethylene centrifuge tube with the aid of a glass rod. The buttermilk was harvested and the remaining butter washed for 0.5 min by the same procedure as the churning, after addition of 0.2 ml of 20 mM ammonium acetate (pH 7.0). This latter procedure was repeated as many times as necessary to obtain an almost transparent aqueous phase. This may require as many as 30 washings. All the washings were pooled with the buttermilk and the suspension centrifuged for 40 min at 150 000 $\times g_{av}$ and 4° C. This gave a brown pellet on the top of which a whitish layer was observed. The supernatant was discarded, the whitish layer gently washed away with the ammonium acetate solution and the brown pellet resuspended in 2 ml of the same solution. The same procedure was repeated once, and the final brown pellet suspended in 20 mM ammonium acetate was stored frozen at −70 °C. This preparation will be referred to as milk fat globule membranes.

Mammary tumour virus. A crude virus preparation was obtained from the skimmed milk by centrifugation through 20 % glycerol in the 10 mM Tris · HCl (pH 7.2)/0.1 M NaCl/30 mM EDTA solution; it was further purified by sucrose density gradient centrifugation (Spinco rotor SW 27, $96\,000 \times g_{av}$) according to Schlomm et al. [12] but using the above solution instead of their buffer and a steeper sucrose gradient (20–60 % sucrose, w/v). The upper fatty layer was discarded and the six opalescent bands were collected separately from the top of the tube with a syringe. Their refractive index was determined with an Abbe refractometer. Tables were used to relate refractive index to concentration and concentration to density [13]. Each of these fractions, diluted with the 10 mM Tris · HCl (pH 7.2)/0.1 M NaCl/30 mM EDTA, was centrifuged at 20 °C for 1 h at $96\,000 \times g_{av}$. The resulting pellets, resuspended in 1 ml 20 mM ammonium acetate adjusted to pH 7.3, were pooled with the corresponding fractions of other identical purifications and stored at −70 °C. Each pool was formed with the fractions from 4–5 purifications.

Lipid analysis

Extraction procedures. The samples were extracted by 20 vols. of chloroform/methanol (2 : 1 by volume) and the extracts were freed of non-lipid contaminants by the washing procedure of Folch et al. [14].

Thin-layer chromatographic techniques. All thin-layer chromatographic analyses were carried out on precoated plates of silicagel (Merck) activated for 1 h at 110 °C. The following solvent systems have been used for lipid fractionation and identification: light petroleum/diethylether/acetic acid, 80 : 20 : 1 by volume (I); chloroform/methanol/water, 65 : 25 : 4 by volume (II); chloroform/methanol/acetic acid/water, 60 : 15 : 3 : 1 by volume (III); tetrahydrofuran/methylal/methanol/water/acetic acid, 50 : 30 : 20 : 5 : 6 by volume (IV). Lipids were detected by one of the following reagents: copper acetate reagent [15], ninhydrin reagent for primary amines and molybdenum blue reagent for phospholipids [16]. Quantitative estimation followed by photodensitometric measurements according to Fewster et al. [15], using the flying spot Vitatron TLD photodensitometer (Vitatron Instruments, Dieren, Holland). With duplicate spotting of both the standard and the sample, the error of the measurement was less than 5 %.

Cholesterol estimation. The unesterified cholesterol was measured photodensitometrically after thin-layer chromatography of the extract in the solvent system I.

Phospholipid analysis. The lipids extract was resolved into three fractions according to Rouser et al. [17] by passage onto a small column (0.7 × 2.5 cm) of DEAE-cellulose and eluting with 10 vols. of chloroform/methanol, 9 : 1 by volume, followed by 10 vols. of chloroform/acetic acid, 1 : 4 by volume, and finally 15 vols. of a mixture of chloroform/methanol, 4 : 1 by volume, containing ammonium acetate (50 mM) and 2 % of concentrated aqueous ammonia. This ensured a complete separation of the choline-containing phospholipids from the acidic phospholipids and provided a basis for phospholipid identification. In addition, the phospholipids eluted from the column were identified by comparing their chromatographic behaviour in several solvent systems (II, III and IV) to that of reference phospholipids; specific reagents were also used for the purpose of identification. The total lipid phosphorus of the unfractionated lipid extract and of the eluate from the DEAE-cellulose column was estimated by the method of Raheja et al. [18]. Individual phospholipids were measured by a photodensitometric method [15] after thin-layer chromatography of the various eluates in the solvent system II; as phosphatidylethanolamine cannot be obtained in a single eluate, this latter phospholipid was measured photodensitometrically after thin-layer chromatography of the unfractionated extract in the solvent system III. The sum of the individual phospholipids measured by this method amounts nearly to 100 % of the total phospholipids of the extract as indicated in Table I. The following phospholipids were used as reference compounds : rat liver phosphatidylcholine and phosphatidylethanolamine and bovine brain sphingomyelin were prepared in our laboratory; phosphatidylserine and phosphatidylinositol were supplied by Supelco.

Polypeptide analysis

Gel electrophoresis. Polypeptides were separated by disc gel polyacrylamide electrophoresis in sodium dodecyl sulphate [19] using the low-gel buffer of pH 9.18. Samples (25 and 100 μg protein of each preparation for protein and carbohydrate staining, respectively) were denatured extemporaneously by addition of 0.1 vol. 0.5 M Na_2CO_3 and 0.2 vol. of 2 % aqueous sodium dodecyl sulphate and incubation for 2 min at 37 °C. 0.2 vol. of β-mercaptoethanol and enough bromophenol blue to

mark the migration front were added. Reference molecular weight polypeptides : phosphorylase b from rabbit muscle (Worthington), β-galactosidase from *Escherichia coli* (Boehringer), creatine phosphokinase from rabbit muscle (Boehringer) and ribonuclease A from bovine pancreas (Sigma) were similarly processed at the same time. Samples and standard mixture were loaded without delay onto the gels and upper electrode buffer was layered on top of the samples. Electrophoresis was carried out at 1.5 mA per gel (0.6 × 7 cm, 12.5 % arcylamide) for 1.5 h at room temperature. Polypeptides were stained for 15 h in 0.07 % Coomassie brilliant blue G-250 (C.I. 42655) in acetic acid/methanol/water, 7 : 50 : 43 by volume, and the gels were destained for 72 h in 5 changes of acetic acid/water, 7 : 93 by volume, at room temperature. Carbohydrates were stained according to Glossmann and Neville [20]. Photodensitometric recordings were performed on a Photovolt Corp instrument using a 545 nm filter for the Schiff stain and a 610 nm filter for the Coomassie stain. Molecular weights were determined according to Shapiro et al. [21]. The relative concentration of each polypeptide was estimated by dividing the area under the corresponding peak of the photodensitometric tracing by the sum of the areas under all the peaks. The areas were calculated by multiplying the height of the peak by width at half height. Similar calculations were performed for the glycopeptides stained by the Schiff reagent although they give only a crude estimate of the relative concentration of the oligosaccharide moieties.

Protein concentrations. Protein was estimated according to Lowry et al. [22] with crystalline bovine serum albumin as the standard.

Enzymatic activities

Xanthine oxidase (EC 1.2.3.2) was estimated at 23 °C by spectrophotometric recording of uric acid at 293 nm, according to Kalckar [23], in the presence of 50 mM pyrophosphate buffer at pH 8.30.

Phosphodiesterase I (EC 3.1.4.1) was estimated at 37 °C according to Aronson and Touster [24], except that the reaction was followed directly in the spectrophotometer cuvette and 5 mM Mg^{2+} added to the reaction mixture.

Alkaline phosphatase activity (EC 3.1.3.1) was measured spectrophotometrically at 37 °C in 50 mM Tris · HCl buffer (pH 8,0) with *p*-nitrophenylphosphate as the substrate.

The activity of 5′-nucleotidase (EC 3.1.3.5) on 5′-AMP was measured at 37 °C according to Aronson and Touster [24].

($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was measured at 37 °C as the difference of P_i released from 5 mM Mg^{2+} ATP in a 25 mM Tris · HCl (pH 7.5) buffer in the presence of 50 mM K^+ and 250 mM Na^+ and when the salts are replaced by 0.1 mM ouabain.

Electron microscopy

Milk fat globule membranes and viral preparations (mammary tumour virus and the other sucrose density gradient fractions) were observed after negative staining [25]. The material, suspended in 20 mM ammonium acetate, was mixed with an equal volume of 2 % potassium phosphotungstate (pH 6.8). It was then adsorbed from droplets (5 min adsorption) onto Formvar carbon-coated grids.

For thin sectioning, pelleted milk fat globule membranes were suspended in a drop of agar, then fixed with glutaraldehyde (4 % in cacodylate buffer, pH 7.0) and

OsO_4 (2 %) [26]. After dehydration in an alcohol series they were embedded in Epon. The sections were post-stained with uranyl acetate [27] and lead citrate [28].

RESULTS

The milk fat globule membranes and the six bands obtained from the crude virus preparation by sucrose density gradient centrifugation (d = 1.12, 1.14, 1.16, 1.17, 1.18 and 1.19 g/cm^3) were examined under the electron microscope and submitted to biochemical analysis.

Electron microscopy

The milk fat globule membrane preparation was very homogeneous, the viral contamination low. It showed large pieces of membranes as tubes or as flat sheets. Sometimes round structures (about 3 μm in diameter) could be observed; they probably correspond to an entire fat globule envelope. These membranes had a loose reticular aspect; they were smooth in profile (Fig. 1). However, some large areas were clearly different: they bore projections and showed numerous empty budding figures typical of viral envelopes (Fig. 2). Thin sections of the milk fat globule membranes (Fig. 3) showed small vesicles limited by a typical double-layered structure, together with pieces of membrane which had a gray amorphous material on one side of the double-layered structure [44, 69].

The fraction d = 1.17 g/cm^3 was composed of typical mammary tumour virus virions (Fig. 4a) : the particles were complex in shape and frequently presented a tail-like structure; they bore characteristic projections organised either on a triangular (Fig. 4b) or a hexagonal (Fig. 4c arrow) pattern. The central core was often distinguishable as a thicker area (Fig. 4a, arrow).

The fraction d = 1.12 g/cm^3 was characteristically composed of small aggregates (around 15 nm in diameter, Fig. 5); it contained some smooth, round vesicles which were otherwise mainly found in fraction d = 1.14 g/cm^3 (Fig. 6a). These empty, collapsed vesicles were heterogeneous in size (from 100 to 500 nm in diameter) and presumably derived from the viral envelope, since many intermediate forms were

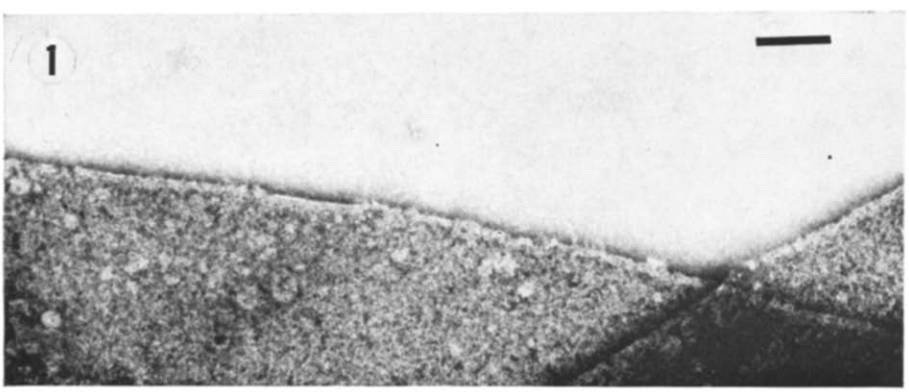


Fig. 1. Electron micrograph of negatively stained milk fat globule membrane, typically smooth in profile. The bar indicates 200 nm.

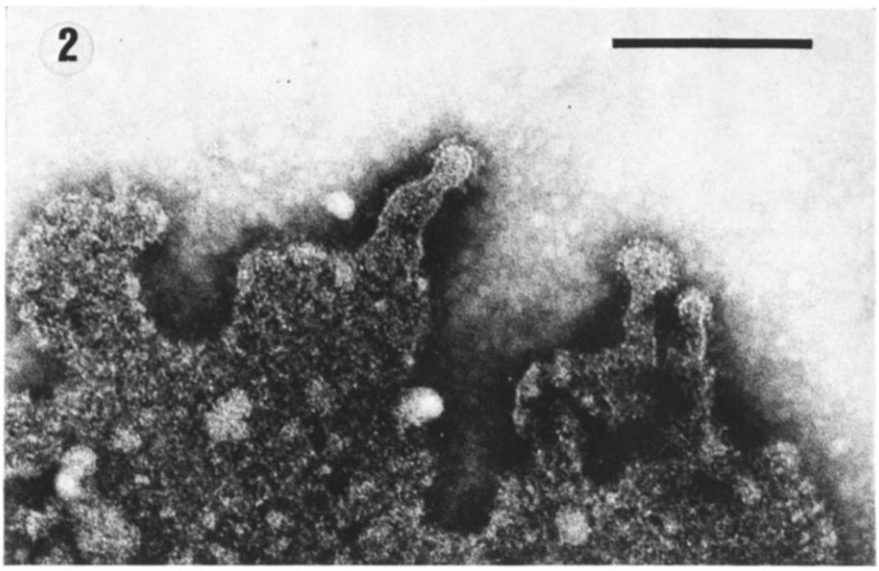


Fig. 2. Electron micrograph of negatively stained milk fat globule membranes presenting, in differentiated areas, budding figures of viral envelopes bearing projections. The bar indicates 200 nm.

observed. The latter bore more or less numerous projections, and retained in some parts of their surface the typical shape and aspect of the virion (Fig. 6b). Sometimes the picture suggested that these vesicles were composed of two concentric structures.

Fraction $d = 1.16$ g/cm^3 was more heterogeneous: it contained the very elongated viral forms (Fig. 7a), when they were present in the starting material, together with viral structures smaller than the normal virions (Fig. 7b). Both forms possessed a typical viral envelope and their banding at a lower density may result from a higher envelope : core ratio. Some mammary tumour virus virions deprived of projections were found in this fraction as well as in denser fractions.

Fractions $d = 1.18$ and 1.19 g/cm^3, denser than the band containing typical virus, were also easily recognisable morphologically. They contained numerous

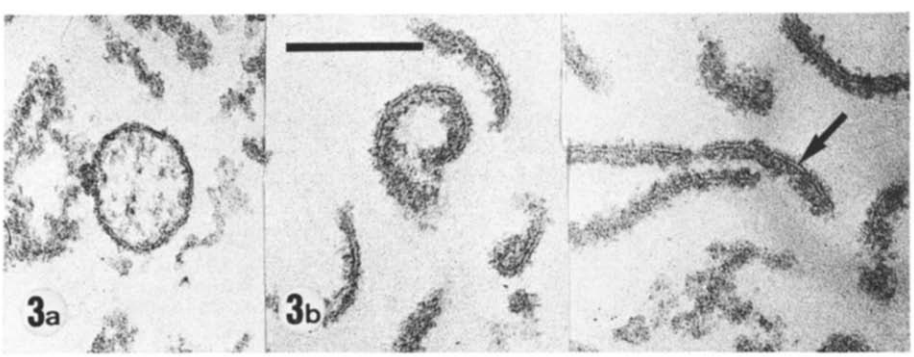


Fig. 3. Electron micrographs of thin sections in pelleted milk fat globule membranes. The double-layered membrane (arrow) is observed either in vesicles (a) or in fragments bearing amorphous material on one side (b). The bar indicates 200 nm.

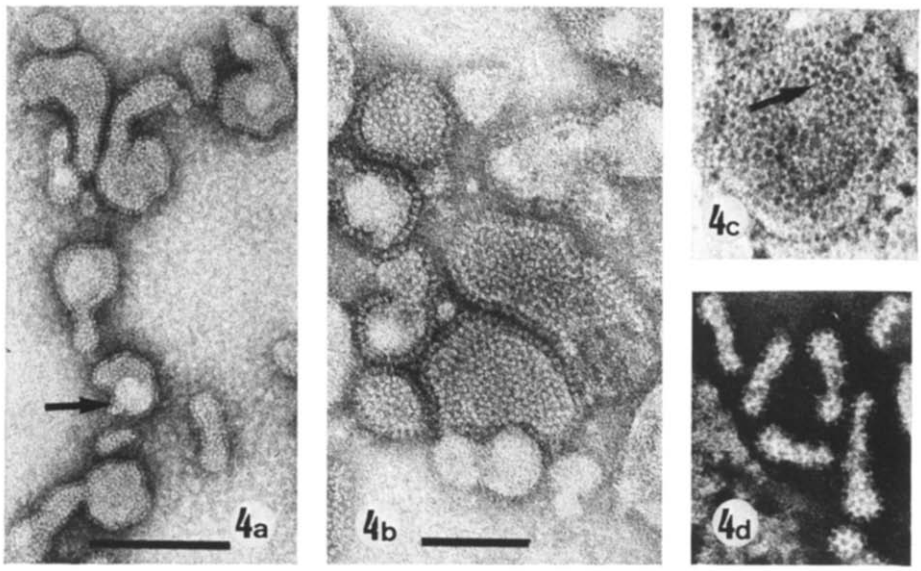
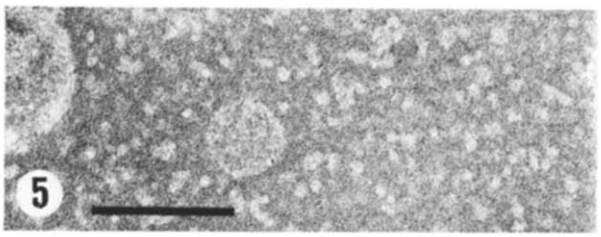


Fig. 4. Electron micrographs of the different sucrose density gradient fractions of the crude virus preparation, after negative staining. The bar indicates 200 nm in Fig. 4a and 100 nm in Figs. 4b, c and d. Fraction $d = 1.17$ g/cm^3 contains typical virions (a); the arrow points to a thicker area revealing a central structure. The envelopes show projections organised either on a triangular pattern (b) or on a hexagonal pattern (c and d). The arrow (4 c) points to the centre of a hexagon.

virions which, penetrated by the stain, clearly displayed their central core (Fig. 8a, mean diameter of the core = 90 nm). The envelopes were frequently broken, and viral debris had a typical appearance (Fig. 8a). On some images, a core-limiting structure was observable (Fig. 8b). Mainly in fraction $d = 1.19$ g/cm^3, viral envelopes degraded in a characteristic manner were observed : they appeared as flat, enlarged membranes, some completely devoid of projections and virus particle shape. They had a reticular appearance and possessed some affinity for the negative stain (Fig. 8c).

Lipid analysis

Data on lipid analysis of the six fractions isolated from the crude virus preparation are presented in Table I and compared with data for the milk fat globule membranes. Milk fat globule membranes and mammary tumour virus ($d = 1.17$ g/cm^3) possessed some common chemical features: in both structures phosphatidylethanolamine was the major phospholipid constituent and the content in sphingomyelin and phosphatidylserine was fairly elevated; moreover, no phosphatidylinositol could be found in the virus preparation while only a minute amount of the latter phospholipid was detected in milk fat globule membranes. Both preparations exhibited similar values of the phosphatidylcholine+sphingomyelin : phosphatidylethanolamine+phosphatidylserine ratio. Differences in the lipid profiles of these two structures involved principally their relative cholesterol and phosphatidylcholine contents (Table I). The cholesterol : phospholipid molar ratio was found to be higher in the virus than in the milk fat globule membranes; moreover, of the total phospholipid of



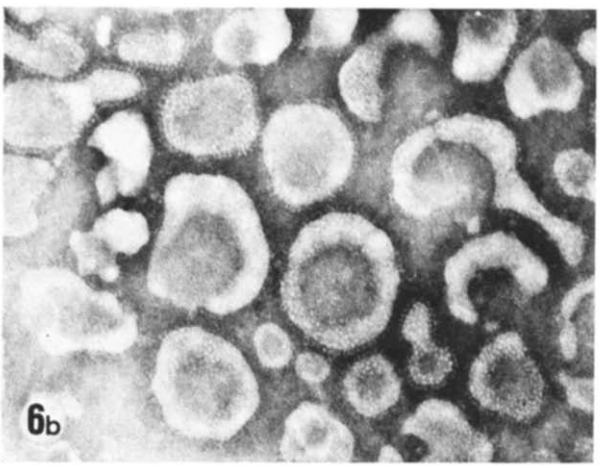
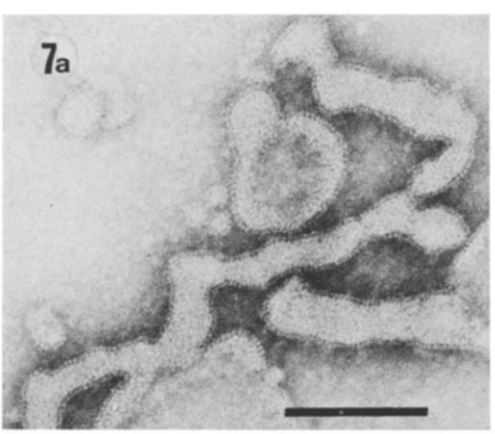


Fig. 5. Electron micrographs of the different sucrose density gradient fractions of the crude virus preparation, after negative staining. The bar indicates 200 nm. Fraction $d = 1.12$ g/cm^3 is composed of small aggregates.

Fig. 6. Electron micrographs of the different sucrose density gradient fractions of the crude virus preparation, after negative staining. The bar indicates 200 nm. Fraction $d = 1.14$ g/cm^3 contains smooth vesicles (a); in some preparations, forms intermediate between the viral envelope and the smooth vesicles are observed (b).



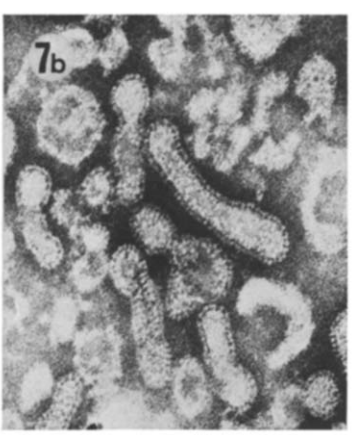


Fig. 7. Electron micrographs of the different sucrose density gradient fractions of the crude virus preparation, after negative staining. The bar indicates 200 nm. In fraction $d = 1.16$ g/cm^3 are found the elongated viral forms (a) and the small viral forms (b).

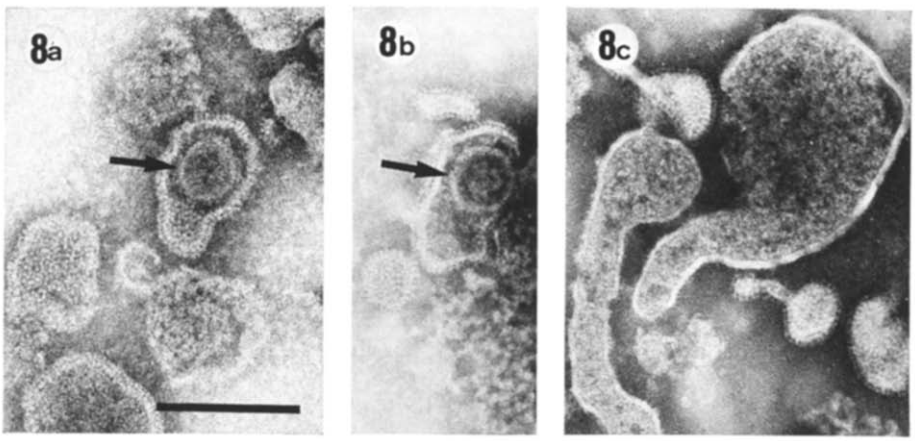


Fig. 8. Electron micrographs of the different sucrose density gradient fractions of the crude virus preparation, after negative staining. The bar indicates 200 nm. Fractions $d = 1.18$ and 1.19 g/cm^3 are characterized by the presence of stain-penetrated virions showing the central core (a and b, arrow) and by the presence of degraded viral envelopes (c). In b, a structure limiting the central core can be distinguished.

the milk fat globule membranes nearly 30 % was accounted for by phosphatidylcholine, while the corresponding value for mammary tumour virus was only 16 %.

Three other fractions ($d = 1.16$, 1.18 and 1.19 g/cm^3) were characterized by a lipid composition very similar to that of the main virus preparation ($d = 1.17$ g/cm^3), e.g. they strongly differed from the milk fat globule membranes in their low value of the phosphatidylcholine : sphingomyelin ratio and higher value of the cholesterol : phospholipid ratio. Two other fractions of lower densities exhibited somewhat intermediate values (Table I); these latter fractions differed from the typical virus preparations by having a lower proportion of phosphatidylethanolamine and a greater proportion of phosphatidylcholine. They were also characterized by their phosphatidylcholine+sphingomyelin : phosphatidylethanolamine+phosphatidylserine ratio, which had a much higher value than that of any other fraction.

Non-polar lipids other than cholesterol have not been quantitated; however, thin-layer chromatography of the lipids extracted from milk fat globule membranes and mammary tumor virus in system I showed that the former contains significant amounts of triglycerides and free fatty acids; cholesterol esters were present only in trace amount in both types of preparation.

Other preparations of milk fat globule membranes and mammary tumour virus ($d = 1.17$ g/cm^3) have been similarly analysed and, although some variations in the cholesterol : phospholipid ratio were observed, the essential analytical features described above were found to be reproducible.

Protein analysis

1 ml of milk yielded an average of 0.43 mg of milk fat globule membrane protein. The respective protein yields per ml of milk for the six sucrose density gradient fractions were 0.10, 0.18, 0.14, 0.13, 0.06 and 0.08 mg, values given by order of decreasing density of the fractions.

TABLE I

LIPID ANALYSIS OF MILK FAT GLOBULE MEMBRANES AND OF THE FRACTIONS OF THE SUCROSE DENSITY GRADIENT CENTRIFUGATION OF THE CRUDE VIRUS PREPARATION

PC, phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; n.d., not detected.

	Cholesterol : mg protein	Phospholipid : mg protein	% of total phospholipid					% of phospholipid recovery*	PC : SM	PC+SM : PE+PS	Cholesterol: P-Lip. (mol/mol)
			PC	SM	PE	PS	PI				
Milk fat globule membrane	0.03	0.12	32.8	12.5	39.8	10.8	3.6	99.5	2.62	0.90	0.43
Viral fractions: (d in g/cm^3)											
1.12	0.04	0.13	37.5	31.3	22.6	8.8	n.d.	102	1.20	2.19	0.70
1.14	0.18	0.39	31.6	30.1	29.0	9.3	n.d.	107	1.04	1.63	0.93
1.16	0.14	0.41	19.0	34.9	34.5	12.5	n.d.	103	0.55	1.12	0.70
1.17 (mammary tumour virus)	0.18	0.26	15.9	26.5	41.6	16.3	n.d.	100	0.60	0.73	1.34
1.18	0.17	0.30	18.4	35.5	36.1	9.9	n.d.	109	0.51	1.17	1.12
1.19	0.15	0.23	19.9	33.2	38.4	8.4	n.d.	98.5	0.60	1.13	1.29

* Sum of the individual phospholipids as % of total phospholipids in the original extract.

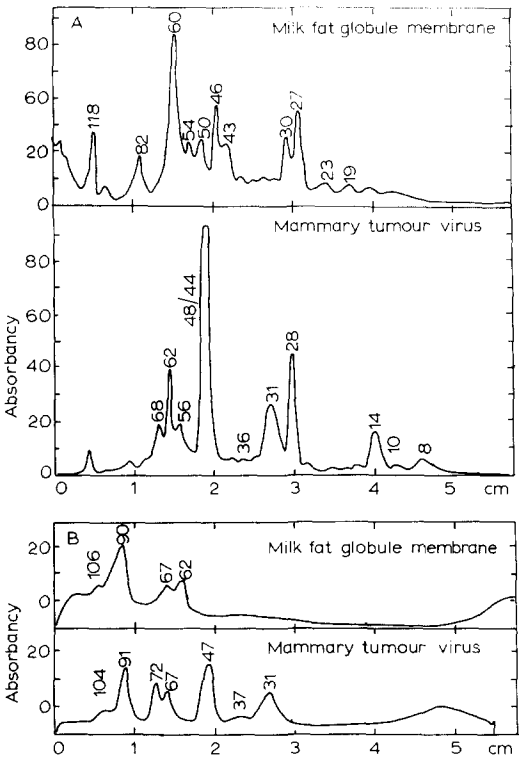


Fig. 9. Photodensitometer tracings of polyacrylamide gel electrophoresis of the polypeptides and glycopolypeptides of milk fat globule membrane and of mammary tumour virus. (A) Polypeptides, (B) glycopolypeptides. Molecular weights are given $\times 10^{-3}$. Ordinates, absorbancy in arbitrary units; abscissae, migration from the origin, in cm.

The polypeptide and glycopolypeptide patterns of milk fat globule membranes and mammary tumour virus were clearly different (Fig. 9).

Milk fat globule membrane contained 22 polypeptides. The 13 major polypeptides accounted for 87 % of the total polypeptide content. Two of them, p67 and p60, apparently corresponded to gp67 and gp62, which together accounted for 40 % of the Schiff-positive material. The remaining two glycopolypeptides, gp106 and gp90, may correspond to the minor polypeptides pl01 and p85 (Table II).

Mammary tumour virus (fraction $d = 1.17$ g/cm^3) contained 19 polypeptides. The 10 major polypeptides accounted for 88 % of the total polypeptide content. Three of these, p62, p48/44 and p31, apparently corresponded to gp67, gp47 and gp31, which together represented 50 % of the total Schiff-positive material of the virus. Three other glycopolypeptides, gp104, gp91 and gp37, might correspond to the minor polypeptides p102, p92 and p36. The individual relative percentages of p48 and p44 on the one hand and gp104 and gp91 on the other were not calculated, as the separation of the latter components was unsatisfactory (Table II).

Three other fractions obtained from the crude viral preparation ($d = 1.16$, 1.18 and 1.19 g/cm^3) were characterized by patterns closely similar to that of the virus (Figs 10 and 11). The fractions ($d = 1.12$ and 1.14 g/cm^3) displayed less re-

TABLE II

POLYPEPTIDE AND GLYCOPOLYPEPTIDE COMPOSITION OF MAMMARY TUMOUR VIRUS AND MILK FAT GLOBULE MEMBRANE

Results are expressed as means of three determinations ± standard deviation. Major polypeptides are underlined.

Mammary tumour virus				Milk fat globule membrane			
Polypeptides		Glycopolypeptides		Polypeptides		Glycopolypeptides	
Molecular weight × 10^{-3}	% total content	Molecular weight × 10^{-3}	% total content	Molecular weight × 10^{-3}	% total content	Molecular weight × 10^{-3}	% total content
121.0±1.0	0.7±0.2			159.0±3.7	5.2±0.8		
102.0±1.0	0.2±0.1	104.2±4.3	31.0±4.6	135.0±3.4	1.4±0.7		
92.0±3.6	1.0±0.1	91.2±3.8		118.0±0.4	6.0±1.6		
74.9±3.6	1.7±0.1			101.2±4.2	0.9±0.4	106.7±0.2	13.9±5.6
68.3±0.6	5.4±0.2	72.2±1.7	13.9±1.6	85.3±0.8	0.1±0.1	90.4±0.4	46.3±4.0
62.8±0.6	5.9±0.2	67.2±1.0	8.1±1.2	82.2±0.4	3.8±0.4		
59.6±0.8	1.8±0.4			77.7±1.2	0.3±0.2		
56.5±0.2	2.8±0.8			71.1±0.3	0.3±0.1		
48.2±0.3	41.3±4.9	47.0±1.0	24.2±3.8	67.0±0.5	2.4±0.7	67.5±1.1	17.8±1.9
44.6±0.7				60.3±1.0	18.0±3.4	62.0±0.4	21.9±1.6
36.7±0.9	1.0±0.5	37.0±1.0	4.5±0.8	54.4±0.4	6.1±1.6		
31.5±0.6	12.3±2.8	31.9±0.9	18.3±2.0	50.4±0.4	6.1±2.6		
28.0±1.8	11.2±3.0			48.7±0.1	1.1±0.9		
25.5±1.1	0.5±0.2			46.3±0.7	9.7±1.3		
20.0±0.6	0.5±0.2			43.0±0.5	2.4±1.3		
16.0±0.5	0.8±0.2			39.3±0.7	1.8±1.0		
14.0±0.5	4.6±1.5			35.6±0.2	1.3±0.8		
10.0±0.7	1.4±0.2			30.3±0.2	10.1±1.5		
7.9±0.8	3.6±0.6			27.0±0.3	10.2±1.6		
				23.5±0.3	4.2±1.4		
				19.5±0.2	3.1±1.0		
				12.2±0.6	1.2±0.5		

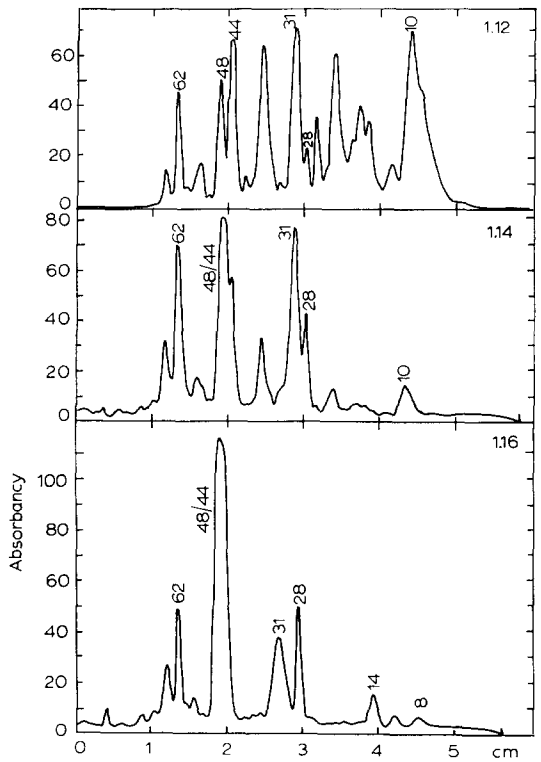


Fig. 10. Photodensitometer tracings of polyacrylamide gel electrophoresis of the polypeptides of the fractions of the sucrose density centrifugation of the crude virus preparation. 1.12, 1.14, 1.16: densities of the fractions in g/cm^3. Ordinates, absorbancy in arbitrary units; abscissae, migration from the origin in cm. Molecular weights of the polypeptides are given $\times 10^{-3}$.

producible patterns, distinctly different from those obtained with virus and milk fat globule membranes (Fig. 10). The relative concentration of eight major and two minor polypeptides of the virus in each fraction are represented in Fig. 12, those of the Schiff-positive material of the glycopolypeptides in Fig. 13. The relative concentration of p68, p62, p56, p36 and gp67 in fraction $d = 1.17$ g/cm^3 was low. Conversely, p48/44, p28, p14, p8, gp47 and gp31 were present at high relative concentrations in this same fraction. The highest concentration of p121 and gp104/91 was found in fraction $d = 1.19$ g/cm^3. The greatest concentrations of p68, p62, p56, p36, gp67 and gp37, as well as the lowest concentrations of p121, p48/44, p28, p14, p8, gp104/91 and gp47, were observed in the two upper fractions ($d = 1.12$ and 1.14 g/cm^3). The relative concentration of gp72 was fairly variable from fraction to fraction. The other polypeptides and glycopolypeptides were present at almost constant relative concentrations.

A diffuse Schiff-positive band, frequently observed near the end of the gels of milk fat globule membranes and virus, even when other concentrations of acrylamide were used, was provisionally attributed to glycolipids.

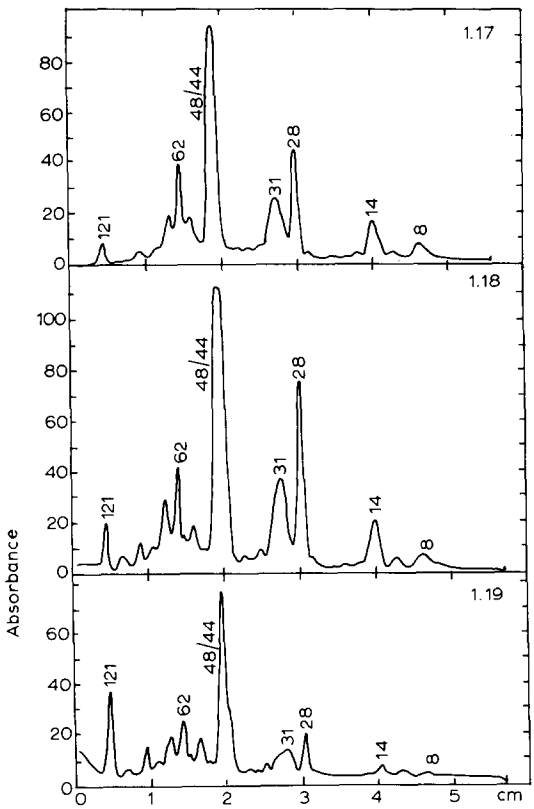


Fig. 11. Photodensitometer tracings of polyacrylamide gel electrophoresis of the polypeptides of the fractions of the sucrose density gradient centrifugation of the crude virus preparation. 1.17, 1.18, 1.19: densities of the fractions in g/cm^3. Ordinates, absorbancy in arbitrary units; abscissae, migration from the origin in cm. Molecular weights of the polypeptides are given $\times 10^{-3}$.

Enzymatic activities

The presence of alkaline phosphatase and xanthine oxidase reported in milk fat globule membranes of other species [4, 29], and of three plasma membrane markers [30] was investigated.

Alkaline phosphatase was absent in milk fat globule membrane preparations and in crude virus preparations (less than 2 nmol *p*-nitrophenol released per h with 50 μg/ml membrane protein).

Xanthine oxidase was present at high specific activities of 0.27, 0.36, 0.57 and 0.82 μmol/min per mg protein in milk fat globule membranes, but viral preparations were all found to be devoid of activity.

$(Na^+ + K^+)$-ATPase was absent from both preparations (less than 2 nmol P_i/h with 200 μg protein).

The *p*-nitrophenylthymidylate phosphodiesterase I was easily detected in several milk fat globule membrane preparations and the specific activities amounted to 2.3, 4.5, 6.3 and 7.0 μmol/h per mg protein at 37 °C. Traces might be present in crude viral preparations.

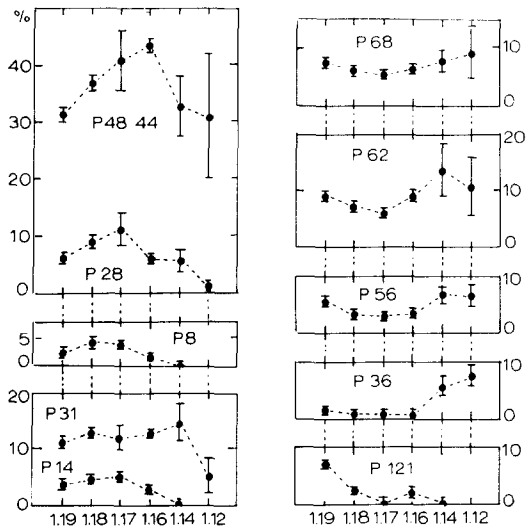


Fig. 12. Relative concentration of 8 major and 2 minor polypeptides in the fractions obtained by sucrose density gradient centrifugation of the crude viral preparation. Ordinates, amount of the polypeptide in the fraction, expressed as % of total polypeptide content of the fraction, mean of three determinations and standard deviation; abscissae, density of the fractions in g/cm^3; p28, etc., polypeptide of molecular weight 28 000.

Low but reproducible activities of 5′-nucleotidase were detected in some milk fat globule membrane preparations, as well as in crude and purified virus (less than 0.5 μmol/h per mg protein).

DISCUSSION

Milk fat globule membrane

While milk fat globule membrane preparations isolated from bovine milk have been extensively studied in several laboratories [3, 4, 31–38], the present work constitutes the first study on murine milk.

The filiation of the milk fat globule membrane with the plasma membrane has been postulated on the basis of morphological and chemical studies [1–3, 35, 36].

The smoothness of the greater part of the surface of our milk fat globule membranes, the presence of areas differentiated as viral envelopes and the double-layered aspect in thin sections, indicate that the milk fat globule membranes are formed at the surface of cells which are also producing virus. Therefore one may expect the presence of small quantities of specific viral components even in the most purified milk fat globule membrane preparations.

Our milk fat globule membrane preparation is characterized by a cholesterol : phospholipid molar ratio slightly below 0.5, and in that respect differs from a typical plasma membrane preparation, which, according to Coleman and Finean [39], should exhibit a ratio close to unity. However, the cholesterol : phospholipid ratio of our milk fat globule membranes compares reasonably well with that found by others for the bovine milk membrane [37] and falls within the range of most values recorded for

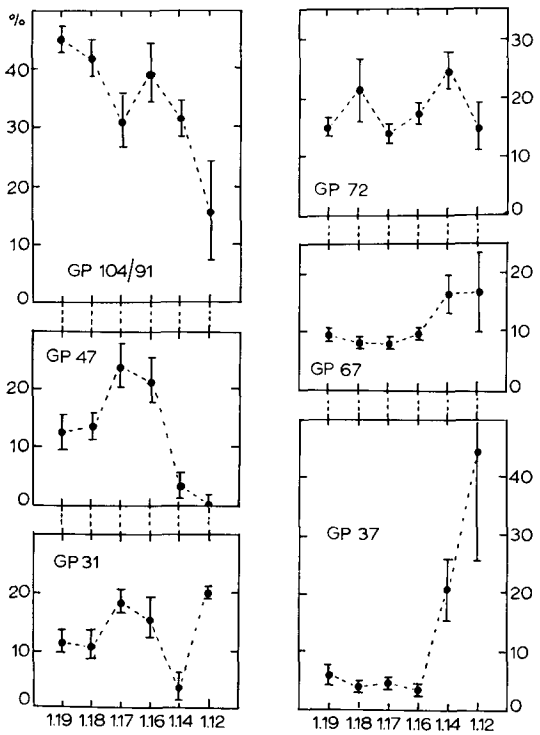


Fig. 13. Relative concentration of the Schiff-positive material of the 7 glycopeptides in the fractions obtained by sucrose density gradient centrifugation of the crude viral preparation. Ordinates, amount of Schiff-positive material of the glycopolypeptide in the fraction, expressed as % of total amount of Schiff-positive material in the fraction; abscissae, density of the fraction in g/cm^3; gp37, etc., glycopolypeptide of molecular weight 37 000.

the cholesterol : phospholipid ratio in the liver plasma membrane [24, 30, 40–43]. As already suggested by others, the milk fat globule membrane may be heterogeneous and may contain some cytoplasmic material [44]; this could account for the rather low value of the cholesterol : phospholipid ratio. The value (0.9) of the ratio of the choline-containing to the non-choline-containing phospholipids found in the milk fat globule membrane preparation is characteristic of plasma membrane [45].

Additional evidence suggesting that milk fat globule membrane is at least partially composed of units derived from the plasma membrane is provided by the presence in our preparation of marker enzymes specific to plasma membrane [30, 46], e.g. phosphodiesterase I and 5'-nucleotidase, and by their complex polypeptide pattern, expected for a structure derived from at least one membrane species [30, 47]. This pattern is as complex as the one recently described for bovine milk membranes [31] but more complex than those previously reported for the bovine [36, 48, 49] and the human [33] milk fat globule membrane. Since no specific measures were taken during the preparative steps to stabilize the enzymatic activities (hardly controllable for xanthine oxidase at least), a 3-fold variation of enzymic activities in different milk fat globule preparations is not unexpected and does not contradict the reproducibility displayed by the polypeptide and glycopolypeptide pattern (Table II).

Mammary tumour virus

The purified preparation of mammary tumour virus ($d = 1.17$ g/cm^3) shows virus particles which have the typical aspect of particles penetrated [50] and not penetrated [51] by the stain. On these particles the projection may appear to be organized on a hexagonal pattern; this feature is not characteristic of damaged or fixed particles, contrary to the suggestion of Sarkar and Moore [51].

The elevated cholesterol : phospholipid molar ratio found in mammary tumour virus preparations compares favorably with the values reported for many enveloped RNA viruses, which are characterized by a ratio close to unity and occasionally greater than one [52–54]. One may conceive that the relative abundance of cholesterol is related to the stability of the viral envelope. The value (0.7) of the ratio of the choline-containing to the non-choline-containing phospholipids found in the virus is consistent with the hypothesis that the viral envelope derives from the host cell membrane [45].

The polypeptide and glycopolypeptide composition of our viral preparation is fairly similar to that reported by others [55–59]. Five of our major proteins (gp47, gp31, p28, p14 and p8) correspond to the five major components described by other workers [58, 59]; however, some differences in the relative proportion of these protein components are apparent in our preparation as compared with the virus studied in other laboratories, namely the concentration of gp72, gp67 and gp47 is higher, and that of p28, p14 and p8 is lower in our preparation. We also noted the presence of two as yet unreported glycopeptides (gp104 and gp91); these might correspond to the high molecular weight glucosamine-labelled substance of Teramoto et al. [59]. In addition, our electrophoretic technique resolves two glycopeptides (gp36 and gp31) in the 30 000 dalton region where heterodisperse gp36 has been reported [59]. It is therefore apparent that the number of polypeptides and glycopolypeptides described in the present paper is greater than that reported in previous studies on the same virus; however, such a high number of protein components has occasionally been observed in other oncogenic viruses [60]. Discrepancies between our results and the data from the literature may result from the use of different mouse strains, virus sources and detection techniques. Nonetheless we obtained similar results with mammary tumour virus purified from the milk of RIII mice (unpublished observation).

Milk fat globule membrane as compared to mammary tumour virus

Although milk fat globule membrane and mammary tumour virus differ to some extent in their phospholipid composition, both structures have a relatively high content of sphingomyelin and of phosphatidylserine; they also exhibit a choline-containing to non-choline-containing phospholipid ratio greater than 0.5 and less than 1.0 like the erythrocyte membrane [45].

As is now well documented, simultaneous high levels of cholesterol and sphingomyelin are characteristics for plasma membranes [30, 43, 61, 62]; in addition, the studies on liver cells suggest that a plasma membrane preparation contains a relatively high content of phosphatidylserine [43, 62]. Therefore our data on the lipid composition of the virus and, to a lesser extent, on the lipid profile of mouse milk fat globule membrane are in keeping with the hypothesis that these latter structures derive from a plasma membrane.

The mammary tumour virus differs from milk fat globule membrane mainly by

having a higher cholesterol content and by containing proportionally more sphingomyelin and less phosphatidylcholine. Interestingly, similar differences have been observed during the course of comparative studies on the lipid composition of some viruses with that of the plasma membrane of their host cells. It has been shown indeed that the cholesterol : phospholipid ratio found in the plasma membrane of the host may sometimes be greatly inferior to that found in the virus [52, 53] and that the virus may contain proportionally less phosphatidylcholine and more sphingomyelin than the plasma membrane of its host cell [52]. The various interpretations which have been advanced to account for such compositional differences between a virus and the membrane presumed to act as a lipid matrix for virus final assembly will be found in some recent papers [52–54]. An additional complication in such comparative studies may arise from the fact that the lipid composition of the membrane of infected cells might be different from that of the non-infected cells [53]; in that respect, it must be noted that we do not know from which population of mammary cells our samples of milk fat globule membrane originates, although electron microscopy indicates that they come at least partially from infected cells, since some membranes display typical viral structures in limited areas. Further investigations will be devoted to comparing the chemical composition of the milk fat globule membrane isolated from the milk of infected mice with that of the milk fat globule membrane prepared from the milk of mice not producing virus and with plasma membranes isolated from the mammary cells.

Mammary tumour virus and milk fat globule membrane were found to have significantly different protein compositions; the possibility remains, however, that both structures might share some common protein components.

The divergency in protein composition between the two structures does not seriously contradict the morphological observation [7] of a common budding from the same cell species. Viral completion might indeed incorporate only viral-coded proteins from the plasma membrane, and disparity of budded membranes would not be more unlikely than disparity among different regions of the plasma membrane of polar cell species as observed in the kidney tubule [63], the renal collecting duct [64], the eel electroplax [65] and the hepatocyte [66].

Fractions from the sucrose density gradient centrifugation of the crude virus preparation

To our knowledge, besides fraction $d = 1.17$ g/cm^3, the viral fractions obtained by sucrose density gradient centrifugation have not been investigated so far for their chemical composition and their morphological appearance.

The material in the denser bands ($d = 1.16$–1.19 g/cm^3) appears to reflect the heterogeneity of the virus as it is produced by the cells, because long and short forms have a somewhat lower density than the typical virion and particles penetrated by the stain have a higher density owing to partial dehydration. Degraded envelopes are also observed in fraction $d = 1.19$ g/cm^3 : these might have lost relatively more lipids than other materials, as indicated by the lipid composition of this fraction. The lipid and protein analyses of the four dense fractions, together with the morphological observations, indicate that these fractions consist entirely of viral material not contaminated by membrane material of other sources.

Fraction $d = 1.14$ g/cm^3 contains smooth envelopes, a fact which is in agreement with the relatively high lipid content that might result from a loss of polypeptide

or glycopolypeptide. These smooth envelopes have a low content in gp47 and gp31 and in that respect resemble the spikeless particles of Cardiff et al. [67], which have the same density and are devoid of the same two glycopeptides. The polypeptide and glycopolypeptide patterns of fraction $d = 1.14$ g/cm^3 share some characteristics with those of the adjacent fractions; moreover, its lipid composition is intermediate between those of fractions $d = 1.12$ g/cm^3 and $d = 1.16$ g/cm^3, as exemplified by the values for the phosphatidylcholine : sphingomyelin ratio and the phosphatidylcholine + sphingomyelin : phosphatidylethanolamine + phosphatidylserine ratio of these fractions.

Fraction $d = 1.12$ g/cm^3 is mainly formed of small aggregates. Its polypeptide and glycopolypeptide profiles are different from both milk fat globule membranes and virus patterns.

The comparative analysis of these fractions leads to the following observations.

(1) The sucrose density gradient centrifugation separates different kinds of virus particles as determined by their chemical composition and their morphology. In that respect it gave, in our hands, better results than Ficoll-^2H_2O [68].

(2) The progressive and reproducible elevation of the relative concentration of p121 and gp104/91 with increasing buoyant density suggests that heavy molecular weight polypeptides and glycopolypeptides are not mere aggregates, and might indicate that these constituents belong to the core of the viral particle.

(3) Because of the parallelism in the variations of their relative concentration, p36 and gp37 on the one hand and p62 and gp67 on the other hand might correspond in each case to a single component; this may also apply to p48 and gp47. Purifications of these peptides are presently being carried out.

(4) The relative concentrations of gp67 and gp37 parallel that of phosphatidylcholine and are strikingly high in fraction $d = 1.12$ g/cm^3, where very few membranes are observed.

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REFERENCES

1 Bargmann, W. and Knoop, A. (1959) Z. Zellforsch. Mikrosc. Anat. 49, 344–388
2 Hollman, K. H. (1959) J. Ultrastruct. Res. 2, 423–443
3 Wooding, F. B. P. (1972) Experientia 28, 1077–1079
4 Dowben, R. M., Brunner, J. R. and Philpott, D. E. (1967) Biochim. Biophys. Acta 135, 1–10
5 Nandi, S. and McGrath, Ch. (1973) Adv. Cancer Res. 17, 383–414
6 Bentvelzen, P. (1974) Biochim. Biophys. Acta 355, 235–259
7 Nakayama, P. (1968) M. A. Thesis, University of California, Berkeley
8 Feldman, D. G. (1963) J. Nat. Cancer Inst. 30, 477–502
9 Calberg-Bacq, C.-M., François, C., Gosselin, L., Osterrieth, P. M. and Rentier-Delrue, F. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 1183–1184
10 Calberg-Bacq, C. M., François, C., Gosselin, L., Osterrieth, P. M. and Rentier-Delrue, F. (1975) Biochem. Soc. Trans. 3, 296–299

11 Leyten, R. (1969) Doctoral Thesis, Faculteit der Landbouwwetenschappen, Katholiek Universiteit, Leuven
12 Schlom, J., Spiegelman, S. and Moore, D. (1971) Nat. New Biol. 231, 97–100
13 De Duve, C., Buttet, J. and Beaufay, H. (1959) Prog. Biophys. Biophys. Chem. 9, 325–360
14 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
15 Fewster, M. E., Burns, B. and Mead, J. F. (1969) J. Chromatogr. 43, 120–126
16 Dittmer, J. C. and Lefter, R. L. (1964) J. Lipid Res. 5, 126–127
17 Rouser, G., Kritchewski, G., Heller, D. and Lieber, E. (1963) J. Am. Oil Chem. Soc. 40, 425–454
18 Raheja, R. K., Kaur, C., Singh, A. and Bhatia, I. S. (1973) J. Lipid Res. 14, 695–697
19 Neville, Jr., D. M. (1971) J. Biol. Chem. 246, 6328–6334
20 Glossmann, H. and Neville, Jr., D. M. (1971) J. Biol. Chem. 246, 6339–6346
21 Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967) Biochem. Biophys. Res. Commun. 28, 815–820
22 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
23 Kalckar, H. M. (1947) J. Biol. Chem. 167, 429–443
24 Aronson, Jr., N. N. and Touster, O. (1974) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 31, pp. 90–102, Academic Press, New York
25 Brenner, S. and Horne, R. W. (1959) Biochim. Biophys. Acta 34, 103–110
26 Sabatini, D. D., Bensch, K. and Barrnett, J. R. (1963) J. Cell Biol. 17, 19–58
27 Watson, M. L. (1958) J. Biophys. Biochem. Cytol. 4, 475–478
28 Reynolds, E. S. (1963) J. Cell Biol. 17, 208–212
29 Martel-Pradal, M. B. and Got, R. (1972) FEBS Lett. 21, 220–222
30 Emmelot, P., Bos, C. J., Van Hoeven, R. P. and Van Blitterswijk, W. J. (1974) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 31, pp. 75–90, Academic Press, New York
31 Mather, I. H. and Keenan, T. W. (1975) J. Membrane Biol. 21, 65–85
32 Keenan, T. W., Morré, D. J., Olson, D. E., Yunghans, W. N. and Patton, S. (1970) J. Cell Biol. 44, 80–93
33 Martel, M. B., Dubois, P. and Got, R. (1973) Biochim. Biophys. Acta 311, 565–575
34 Bauer, H. (1972) J. Dairy Sci. 55, 1375–1387
35 Farrell, Jr., H. M. and Thompson, M. P. (1971) J. Dairy Sci. 54, 1212–1228
36 Bracco, V., Hidalgo, J. and Bohren, H. (1972) J. Dairy Sci. 55, 165–172
37 Kobylka, D. and Carraway, K. L. (1972) Biochim. Biophys. Acta 288, 282–295
38 Huang, C. M. and Keenan, T. W. (1972) Biochim. Biophys. Acta 274, 246–257
39 Coleman, R. and Finean, J. B. (1966) Biochim. Biophys. Acta 125, 197–206
40 Fleischer, S. and Packer, L. (1974) in Mehods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 31, pp. 6–41, Academic Press, New York
41 Van Hoeven, R. P., Emmelot, P., Krol, J. H. and Oomen-Meulemans, E. P. M. (1975) Biochim. Biophys. Acta 380, 1–11
42 Yamashita, K. and Field, J. B. (1974) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 31, pp. 144–149, Academic Press, New York
43 Van Hoeven, R. P. and Emmelot, P. (1972) J. Membrane Biol. 9, 105–126
44 Wooding, F. B. P. (1972) J. Ultrastruct. Res. 37, 388–400
45 Bretscher, M. S. (1973) Science 181, 622–629
46 Hubbard, A. L. and Cohn, Z. A. (1975) J. Cell Biol. 64, 461–479
47 Wooding, F. B. P. (1973) J. Cell Sci. 13, 221–235
48 Anderson, M., Camston, T. and Cheeseman, G. L. (1974) Biochem. J. 139, 653–660
49 Kitchen, B. J. (1974) Biochim. Biophys. Acta 356, 257–269
50 Calafat, J. and Hageman, P. (1969) Virology 38, 364–368
51 Sarkar, N. H. and Moore, D. H. (1974) Virology 61, 38–55
52 Quigley, J. P., Rifkin, D. B. and Reich, E. (1972) Virology 50, 550–557
53 Blough, H. A. and Tiffany, J. M. (1973) Adv. Lipid Res. 11, 267–338
54 Lenard, J. and Compans, R. W. (1974) Biochim. Biophys. Acta 344, 51–94
55 Witte, O. N., Weissman, I. L. and Kaplan, H. S. (1973) Proc. Natl. Acad. Sci. U.S. 70, 36–40
56 Nowinski, R. C., Sarkar, N. H., Old, L. J., Moore, D. H., Scheer, D. I. and Hilgers, J. (1971) Virology 46, 21–38

57 Smith, G. H. and Wivel, N. A. (1973) J. Virol. 11, 575–584
58 Dickson, Cl. and Skehel, J. J. (1974) Virology 58, 387–395
59 Teramoto, Y. A., Puentes, M. J., Young, L. T. J. and Cardiff, R. D. (1974) J. Virol. 13, 411–418
60 Bolognesi, D. P., Huper, G., Green, R. W. and Graf, T. (1974) Biochim. Biophys. Acta 355, 220–235
61 Keenan, T. W. and Morré, D. J. (1970) Biochemistry 9, 19–25
62 Zambrano, F., Fleischer, S. and Fleischer, B. (1975) Biochim. Biophys. Acta 380, 357–369
63 Wilfong, R. F. and Neville, Jr., D. M. (1970) J. Biol. Chem. 245, 6106–6112
64 Schwartz, I. L., Shlapz, L. J., Kinne-Saffran, E. R. and Kinne, R. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2595–2599
65 Baumann, A., Changeux, J.-P. and Benda, P. (1970) FEBS Lett. 8, 145–148
66 Wisher, M. H. and Evans, W. H. (1975) Biochem. J. 146, 375–388
67 Cardiff, R. D., Puentes, M. J., Teramoto, Y. A. and Lund, J. K. (1974) J. Virol. 14, 1293–1303
68 Nowinski, R. C., Old, L. J., Moore, D. H., Geering, G. and Boyse, E. A. (1967) Virology 31, 1–14
69 Keenan, T. W. and Olson, D. E. (1971) J. Dairy Sci. 54, 294–299