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CHARACTERISATION OF THE SURFACE OF BOVINE MILK FAT GLOBULE MEMBRANE USING MICROELECTROPHORESIS

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SUMMARY

The nature of the ionogenic groups on the surface of the milk fat globule membrane was studied by microelectrophoresis of intact fat globules after chemical and enzymic modification. The changes in pH–mobility curves effected by formaldehyde and 2,4-dinitrofluorobenzene showed that the membrane surface contained amine groups. These were identified as arising from lysine and arginine by chromatography of their dinitrophenyl derivatives. The contribution of *N*-acetylneuraminic acid and phosphate to the surface charge was demonstrated by their specific removal by neuraminidase and phospholipase C, respectively. After removal of *N*-acetylneuraminic acid and phosphate, anionogenic effects remained which were attributed to protein carboxyl groups. These groups could be partially esterified using diazomethane. The effect of sodium dodecyl sulphate and of ionic strength on electrophoretic mobility indicated that the surface contains little neutral lipid and is predominantly ionogenic. The results obtained concerning the nature of the surface of the milk fat globule membrane support the hypothesis that the milk fat globule membrane originates from the plasmalemma of the mammary alveolar cell.

INTRODUCTION

The origin of the milk fat globule membrane has been the subject of considerable discussion in recent years[1–5]. Bargmann and Knoop[6] first suggested that the milk fat globule membrane was identical with the plasma membrane of the secretory cell. Their electron microscopy studies indicated that the fat droplet is formed in the basal region of the secretory cell and migrates to the apical surface, where it bulges out into the alveolar lumen becoming progressively enveloped in the plasma membrane. Finally the membrane is pinched off leaving intact membranes both on the emerged droplet and on the apical cell surface. This mechanism is strongly supported by demonstrations that bovine milk fat globule membrane has a chemical[3] and enzymic[7] content closely resembling that of the plasma membrane of lactating mammary cell. Recent electron micrographs[5] suggest that Golgi vesicles are involved in the extrusion process and that the initially formed milk fat globule membrane is composed of both

Abbreviation; DNP-, dinitrophenyl-.

plasmalemma and Golgi vesicle membrane. Marker enzymes normally associated with Golgi vesicles have since been reported in milk fat globule membrane[8].

While available evidence indicates that milk fat globule membrane consists of a continuous mammalian unit membrane immediately after separation from the secretory cell, its subsequent fate is less clear. Most fat globules in the alveolar lumen or those in expressed milk have a milk fat globule membrane which differs from plasmalemma or the initially formed milk fat globule membrane in electron micrographs. It has been suggested that either a structural rearrangement[3] or a partial fragmentation[9] of initial unit membrane occurs after leaving the secretory cell.

In order to gain information regarding the nature of the milk fat globule membrane in expressed milk, we have studied the electrophoretic mobility of intact milk fat globules. Electrokinetic studies, in particular mobility–pH profiles, have made major contributions to our knowledge of animal and bacterial cell surfaces[10]. However, very little has been published concerning application of this technique to the milk fat globule[11]. In this paper we report the effect of various chemical and enzymic modifications on electrophoretic mobility–pH curves of milk fat globules and relate their effects to the nature of the ionogenic groups on the outer surface of the milk fat globule membrane.

MATERIALS AND METHODS

Chemicals and enzymes

All solutions were made up in glass distilled water. Analar grade formaldehyde and acetaldehyde, also general reagent grade 2,4-dinitrofluorobenzene and *p*-toluenesulphonyl chloride, were obtained from B.D.H. Sodium dodecyl sulphate was obtained in pellet form from Koch-Light laboratories.

Diazomethane was prepared from *N*-methyl-*N*-nitrosotoluene sulphonamide (B.D.H.) as outlined by Vogel[12]. Diazomethane was distilled in ether and collected in ice-cold ethanol. *O*-Methylisourea was obtained as its sulphate from K and K laboratories.

Crystalline synthetic *N*-acetylneuraminic acid was obtained from the Sigma Chemical Co. Neuraminidase Type V (EC 3.2.1.18) from *Clostridium perfringens* was also obtained from Sigma.

Phospholipase C (EC 3.1.4.3) from *C. perfringens* was obtained from Koch-Light.

Isolation of fat globules

Milk from 12 Friesian cows was pooled and maintained within 3–4 °C of the milking temperature in an insulated container. Within 20 min of milking the milk was warmed to 40 °C and separated in an Alfa bench cream separator calibrated to give 40% cream. This cream was diluted with three times its volume of double distilled water and reseparated at 40 °C. The dilution and separation was repeated a further eight times to give cream with a total fat content of 64% which was stored at 4 °C.

Electrophoretic measurements

The electrophoretic mobilities of milk fat globules were measured in a rectangular closed cell thermostatted by a surrounding water jacket at 25 ± 0.2 °C using the

apparatus described by Gitten and James[13]. The cell was mounted in the lateral position of Hartman *et al.*[14]. Apparatus alignment and calibration was carried out using the method outlined by Heard and Seaman[15]. All subsequent measurements were carried out at the nearer stationary layer. Cream was diluted 1:300 (v/v) and a minimum of 20 individual timings (measured to 0.1 s) were used to obtain the mean mobility value.

The following buffers were used: pH 1.5–2.6: HCl and NaCl; pH 2.6–9.6: NaCl, sodium acetate, sodium barbiturate and HCl; and pH 9.6–11.0: NaCl, sodium acetate, sodium barbiturate and NaOH. pH–mobility curves were always determined at I 0.05 unless other wise stated. A Radiometer (Copenhagen) universal pH meter 29 was used to determine the buffer pH and a Radiometer Type CDM 2d conductivity bridge used for conductivity measurements. For measurements of mobility against ionic strength, buffer solutions from 0.01 to 0.1 ionic strength were used.

Methods of treatment

After each chemical or enzyme treatment, cream was removed by centrifugation at $3000 \times g$ for 3 min and then diluted with the appropriate buffer for mobility determinations. Washing with a medium implies the suspension of cream in that medium followed by removal by centrifugation at $3000 \times g$ for 3 min.

Formaldehyde and acetaldehyde. Diluted cream (1:5 in buffer, pH 8.0) containing 2% (w/v) aldehyde was stored at 4 °C. The mixture was shaken gently every day to disperse separated cream.

p-Toluenesulphonyl chloride. Diluted cream (25 ml, 1:5 in buffer, pH 7.5) was stirred with the reagent (50 mg) at 4 °C for 3 h.

2,4-Dinitrofluorobenzene. Cream (5 ml) diluted with ethanol (100 ml) containing the reagent (0.1 ml) and $NaHCO_3$ (0.9 g) was stirred for 5 h at 20 °C (ref. 16). The cream was separated by centrifugation at $3000 \times g$ for 3 min and washed 3 times with ethanol.

O-Methylisourea. Diluted cream (50 ml, 1:5 in buffer, pH 7.0) containing 75 mg reagent was allowed to stand at 20 °C for 1 h.

Diazomethane. Cream was washed with buffer, pH 3.0, and diluted (1:5) with ethanol. A solution of diazomethane in ethanol and diethyl ether was added dropwise until the supernatant appeared yellow in colour and gas ceased to be evolved. The cream was washed with ethanol before mobility determination.

Sodium dodecyl sulphate. Diluted cream (1:5 in buffer, pH 7.0) containing 10^{-6}–10^{-1} M reagent was stored for 30 min at 20 °C.

Neuraminidase. Neuraminidase (0.5 mg enzyme per ml cream) or heat-denatured enzyme was incubated with diluted cream (1:5 in buffer, pH 5.5) for 3 h at 37 °C in a shaking water bath.

Phospholipase C. Phospholipase C (0.5 mg/ml cream) or heat-denatured enzyme was incubated with diluted cream (1:5 in buffer, pH 7.0) for 1 h at 37 °C.

Determination of sialic acid

Colourimetric determination. The subnatant remaining after neuraminidase-treated cream had been removed by centrifugation at $3000 \times g$ was further centrifuged at $10\,000 \times g$ for 15 min. Aliquots of the clear subnatant were taken and purified on an anion-exchange column, AG-1-X8 (Bio Rad) as described by Svennerholm[17]. The

resulting solution was assayed for sialic acid using the Aminoff[18] modification of Warren's[19] thiobarbituric acid method.

Thin-layer chromatography. 500 ml of cream were treated with neuraminidase and the subnatant purified by centrifugation and ion-exchange chromatography as for the colourimetric determination. The resulting solution was chromatographed on cellulose plates (0.25 mm) in *n*-butanol–*n*-propanol–0.1 M HCl (1:2:1, v/v/v).

Both resorcinol and *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) sprays were used to visualise the spots.

Determination of phosphate

Colourimetric determination. The subnatant remaining after phospholipase C-treated cream had been removed by centrifugation at $3000 \times g$ was centrifuged for a further 15 min at $10000 \times g$ and passed through a membrane filter (0.45 μm). A sample of the clear filtrate was ashed with concentrated H_2SO_4 as described by Chen *et al.*[20]. The residue was made up to a volume of 5 ml with distilled water and 1-ml samples assayed for inorganic phosphorus[20].

Identification of dinitrophenyl amino acids

10 ml of cream previously treated with 2,4-dinitrofluorobenzene were washed thoroughly with ethanol and hydrolysed with 50 ml of 5.8 M HCl for 16 h at 100 °C. The hydrolysate was cooled, the solidified fat removed by filtration and the filtrate concentrated (15 ml) on a rotary evaporator. This solution was extracted with ether and ethyl acetate according to the method of Brenner *et al.*[21] and the extracts washed with 0.1 M HCl.

Both ether and acid-soluble fractions were chromatographed on silica gel (0.25 mm). The ether-soluble amino acid fraction was run in chloroform–methanol–glacial acetic acid (95:5:1, v/v/v) and the acid-soluble fraction in *n*-propanol–34% NH_4OH (70:30, v/v).

Dinitrophenyl (DNP-) amino acids ran as yellow spots.

Heat of ionisation

Mobilities were measured at 25 and 35 °C and the bulk pK_a of negative ionogenic groups measured at both temperatures. The heat of ionisation was calculated using the Arrhenius equation.

RESULTS

Electrophoretic mobility–pH relationships

Normal washed milk fat globules. Fig. 1 shows the pH–mobility relationship for milk fat globules which had been washed nine times with distilled water and stored at 4 °C. Very similar curves were obtained for all samples of cream which had been washed more than four times. The step at pH 6–7 was found to be more or less prominent depending on the samples. This could be attributed to the ionisation of a histidine residue (pK_a 5.6–7.0).

Treatment with amine-modifying reagents. Treatment with formaldehyde for 10 days increased the negative electrophoretic mobility over the pH range 2–12 compared with a control (Fig. 2). The positive branch and the step at pH 10–11 were

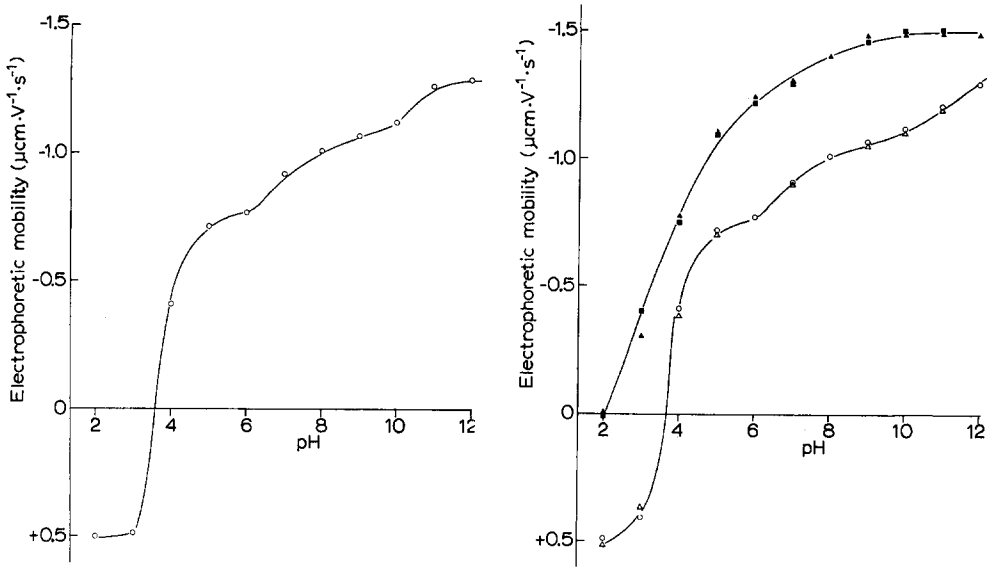

Fig. 1. pH–mobility curve of untreated cream.

Fig. 2. pH–mobility curve of formaldehyde-treated and 2,4-dinitrofluorobenzene-treated cream. ○—○, untreated; ▲—▲, formaldehyde treated; △—△, ethanol treated; ■—■, 2,4-dinitrofluorobenzene treated.

eliminated on formaldehyde treatment. Such modifications are consistent with total blocking of primary amino groups on the surface of the milk fat globule membrane by formaldehyde[22]. The gradual increase in negative mobility of the formaldehyde-treated curve over the pH range 2–5 suggests the discharge of more than one kind of anionic group.

Identical changes in a control curve were brought about by treatment of cream with ethanolic 2,4-dinitrofluorobenzene. That ethanol treatment itself had little effect on the pH mobility curves is shown by the similarity of both 2,4-dinitrofluorobenzene and control curves with the formaldehyde-treated and its control curve, respectively (Fig. 2).

Treatment with *O*-methyl isourea eliminated the step at pH 10–11 which is consistent with the removal of an amine group with a pK_a 10.5 (ref. 23).

Acetaldehyde treatment up to 20 days failed to totally eliminate the positive branch of a normal pH–mobility profile indicating incomplete blocking of surface amino groups. Shaking with an aqueous suspension of *p*-toluenesulphonyl chloride for 3 h similarly failed to block the primary amine groups. Extension of reaction time beyond 3 h resulted in disruption of the membrane as evidenced by butter formation.

Treatment with carboxyl modifying reagents. Treatment with diazomethane decreased the negative electrophoretic mobility over the pH range 4–7 compared with a control which had been exposed to ethanol and ether as shown in Fig. 3. The presence of ether led to partial destabilization of the cream as was evidenced by some butter formation and is also reflected in the shape of the control curve as compared with that

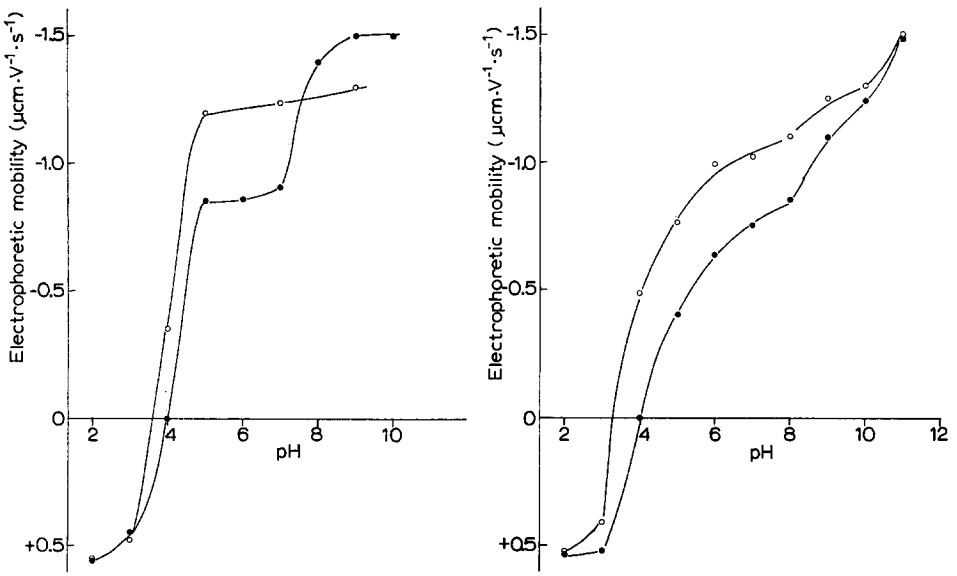

Fig. 3. pH–mobility curve of diazomethane-treated cream. ○—○, ethanol and ether treated; ●—●, diazomethane treated.

Fig. 4. pH–mobility curve of neuraminidase-treated cream. ○—○, treated with denatured enzyme; ●—●, neuraminidase treated.

in Fig. 2. The decrease in mobility over the pH range 4–7 is consistent with the partial removal of the effective charge of carbonyl groups[16].

The increase in negative mobility observed with the diazomethane treated curve above pH 7 is due to hydrolysis of the carboxyl methyl ester re-releasing carboxyl group charges.

Treatment with neuraminidase. Neuraminidase caused a general fall in negative electrophoretic mobility over the pH range 2–11 compared with a control incubated with heat denatured enzyme (Fig. 4). Such a fall is consistent with cleavage of sialic acid molecule and consequent loss of the contribution of their carboxyl groups to surface charge.

Successive formaldehyde and neuraminidase treatment gave the pH–mobility profile shown in Fig. 5. Comparison with a control cream treated with formaldehyde and inactive enzyme indicated that the enzyme has removed anionogenic groups of pK_a 2.5 corresponding to the pK_a of the carboxyl group of sialic acid. The same relationship between the two curves shown in Fig. 5 was obtained when two creams were first treated with active or inactive neuraminidase followed by formaldehyde treatment.

Treatment with phospholipase C. Figs 6 and 7 show the effects of phospholipase C on normal and formaldehyde-treated creams, respectively. As in the case of neuraminidase treatment, the changes observed are consistent with the removal of an ionogenic group pK_a 2.5. In this case the effect would be attributed to the cleavage of the glycerol–phosphate linkage of phospholipid molecules and removal of the phosphate groups from the surface of the milk fat globule membranes.

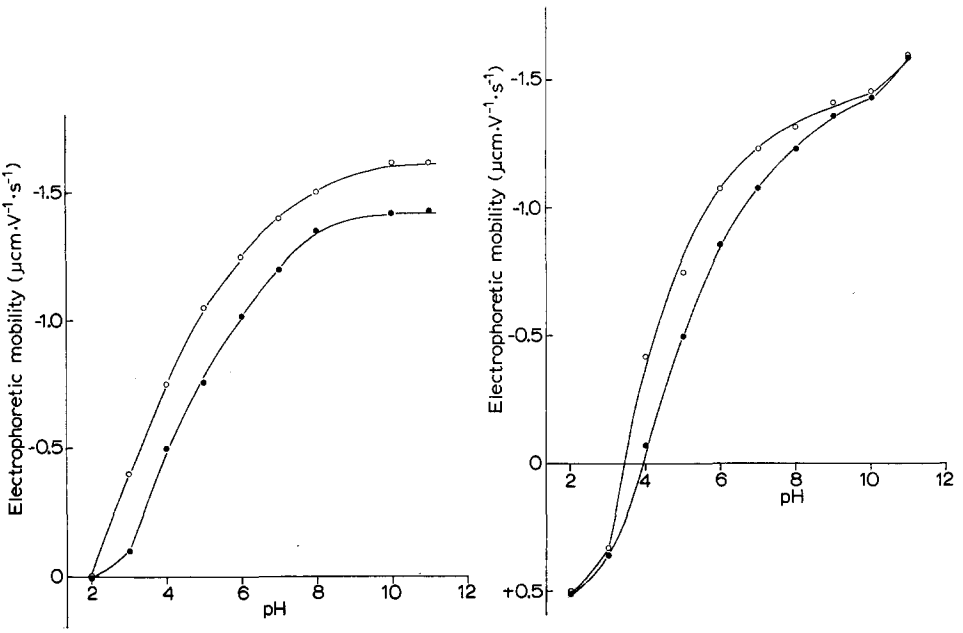

Fig. 5. pH–mobility curve of formaldehyde-blocked cream treated with neuraminidase. ○—○, treated with formaldehyde and denatured enzyme; ●—●, neuraminidase treatment of formaldehyde-blocked cream.

Fig. 6. pH–mobility curve of phospholipase C-treated cream. ○—○, cream treated with denatured enzyme; ●—●, phospholipase *C* treated.

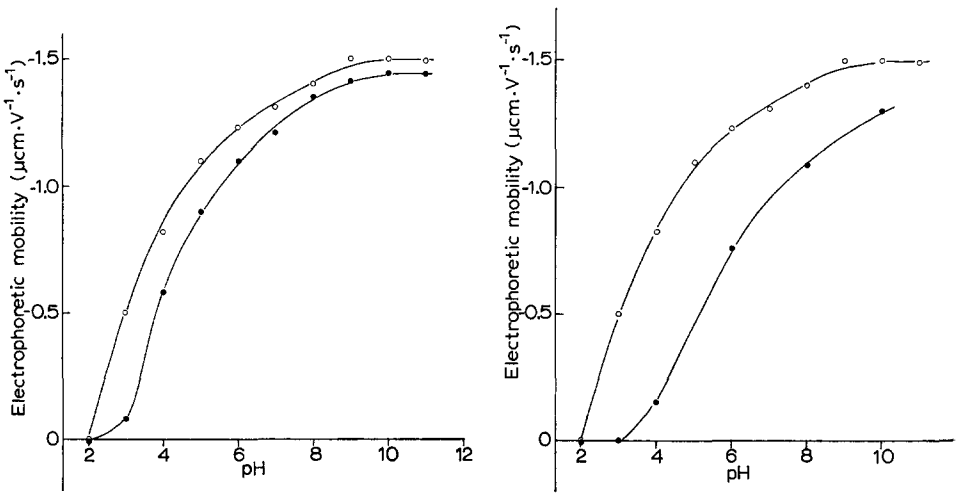

Fig. 7. pH–mobility curve of formaldehyde-blocked cream treated with phospholipase C; ○—○, treated with formaldehyde and denatured enzyme; ●—●, phospholipase C treatment of formaldehyde-blocked cream.

Fig. 8. pH-mobility curve of formaldehyde-blocked cream treated with neuraminidase and phospholipase C. ○—○, treated with formaldehyde and denatured enzymes; ●—●, formaldehyde-blocked cream treated with neuraminidase and phospholipase C.

Treatment of formaldehyde-blocked cream with neuraminidase (Fig. 5) or phospholipase C (Fig. 7) alone failed to totally remove the negative electrophoretic mobility at pH 3.0. When formaldehyde-blocked cream was treated with both neuraminidase and phospholipase C however, mobility at pH 3.0 was reduced to zero indicating that phosphate and sialic acid carboxyls are the only groups contributing to the negative electrophoretic mobility at this pH (Fig. 8).

Treatment with sodium dodecyl sulphate

As shown in Fig. 9, the negative electrophoretic mobility of cream at pH 7.0 was not significantly increased by dodecyl sulphate over the concentration range 10^{-6}–$5 \cdot 10^{-4}$ M. This is consistent with the absence of surface neutral lipid[21]. The increase in mobility shown over the range $5 \cdot 10^{-4}$–10^{-1} M suggests dodecyl sulphate adsorption onto an ionogenic surface, *e.g.* protein[24].

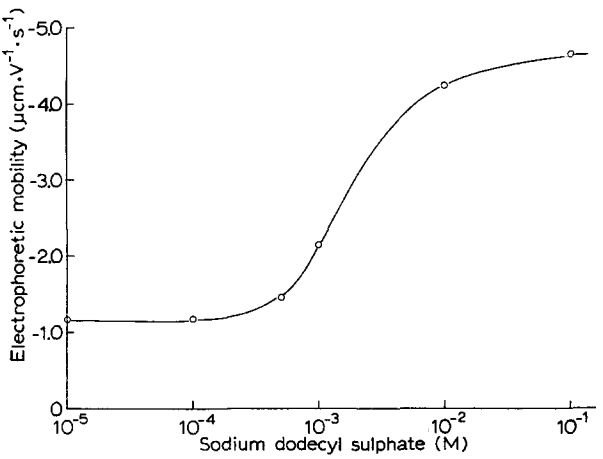

Fig. 9. Variation of mobility with dodecyl sulphate concentration.

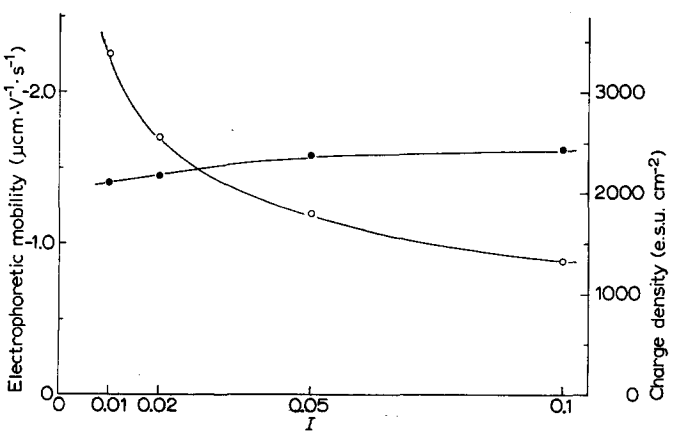

Fig. 10. Variation of mobility and surface charge density with ionic strength. o —o, electrophoretic mobility; ●—●, surface charge density.

Effect of ionic strength

Increasing the ionic strength of the buffer results in a corresponding decrease in electrophoretic mobility at pH 7.0 (Fig. 10). The surface charge density was calculated from electrophoretic mobility values *via* the Smoluchowski[25] equation and remains relatively constant with increasing ionic strength. These curves are again consistent with the predominant ionogenic nature of the milk fat globule membrane surface[26].

Determination of sialic acid

Incubation of cream with neuraminidase released 114.6 ± 3.5 μg of sialic acid per ml of cream as determined by the colourimetric assay. This corresponds to $9.14 \cdot 10^6$ molecules of sialic acid per globule (of mean diameter). Using the fall in negative electrophoretic mobility the corresponding figure is $3.01 \cdot 10^5$ molecules of sialic acid per average size fat globule[27,28]. The ratio of molecules of sialic acid measured chemically to those calculated from the fall in mobility is approx. 30:1. No difference in the amount of sialic acid released by either neuraminidase or dilute acid was found.

Thin-layer chromatography of the released sialic acid showed a single zone with R_F 54 (*n*-butanol–*n*-propanol–0.1 M HCl (1:2:1, v/v/v)) corresponding to that of a standard of *N*-acetylneuraminic acid and significantly different from the reported values for *N*-glycolylneuraminic acid or *N*-acetyl-*O*-acetylneuraminic acids.

Determination of phosphate

Incubation of cream with phospholipase C released 15.36 μg of inorganic phosphate per ml of cream according to colourimetric assay. This would correspond to $3.97 \cdot 10^6$ molecules of phosphate per globule (of mean diameter).

Calculation based on the fall in negative mobility brought about by phospholipase C gave a figure of $1.94 \cdot 10^5$ molecules of phosphate per globule. This is less than the figure obtained for colourimetric assays by a factor of 20.

Identification of amino groups on the surface of the membrane

Treatment of cream with ethanolic 2,4-dinitrofluorobenzene followed by isolation of membrane material and total acid hydrolysis allowed separation of two yellow compounds in the acid-soluble fraction which gave R_F values 39 and 52 on thin-layer chromatography (*n*-propanol–34% NH_4OH, 70:30, v/v), corresponding to standards of DNP-L-arginine and ε-DNP-L-lysine, respectively.

No DNP-amino acids were detected in the ether soluble fraction.

Determination of heat of ionisation

Heat of ionisation determined as described in Materials and Methods gave a figure of 1100 cal/mole.

DISCUSSION

The pH–mobility curve (Fig. 1) for washed cream can be explained on the basis of combined contributions from specific ionogenic groups on the outer surface of the milk fat globule membrane. Thus, the positive branch of the curve was eliminated by the amine-blocking reagents formaldehyde or 2,4-dinitrofluorobenzene. Acid

hydrolysis of 2,4-dinitrofluorobenzene-treated membrane gave DNP-arginine and ε-DNP-lysine indicating that arginine and lysine are responsible for free primary amine groups on the surface.

The step in the pH–mobility curve (Fig. 1) at pH 10–11 can be ascribed specifically to the amine group of the lysine residue since it is removed not only by formaldehyde and 2,4-dinitrofluorobenzene treatment but also by *O*-methylisourea[23]. The latter reagent converts lysine to homoarginine thereby shifting the pK_a of the acid from 10.5 to 12.5.

When either normal or formaldehyde-blocked cream was treated with neuraminidase, changes in the pH–mobility curve were obtained which corresponded to the removal of an ionogenic group with pK_a 2.5. That *N*-acetylneuraminic acid (pK_a 2.6)[29] was in fact being removed by this treatment was confirmed by thin-layer chromatography and colourimetric assay.

Calculation of the number of *N*-acetylneuraminic acid groups per unit surface area of membrane using the fall in negative mobility at pH 7.0 gave a figure 30 times smaller than that obtained from colourimetric assay. Similar discrepancies have been observed in mammalian[28,30] cells and bacteria[31] and have been attributed to a variety of factors. It is possible for instance that some *N*-acetylneuraminic acid molecules are "buried" and do not contribute to the charge at the electrokinetic plane of shear whilst still being accessible to neuraminidase. It is also possible that removal of *N*-acetylneuraminic acid molecules allows previously masked anionogenic groups to become effective at the plane of shear, thus reducing the apparent fall in surface charge density. Inadequacies in the calculation of surface charge density based on electrophoretic mobility[25] (*e.g.* neglecting surface conductance effects and assumptions regarding average size globules) might also account for the lower figure obtained.

Phospholipase C treatment of normal or formaldehyde-blocked cream produced changes in the pH–mobility curve exactly parallel to those obtained with corresponding neuraminidase treatment. Again the changes can be attributed to removal from the surface of an anionogenic group of pK_a 2.5 which would in this case correspond to the phosphate group of phospholipids. Colourimetric determination of cleaved phosphate gave a higher figure than that obtained from mobility measurements probably for the same reasons as those outlined above for sialic acid.

When the contribution made by *N*-acetylneuraminic acid and phosphate to the negative electrophoretic mobility was removed by successive neuraminidase and phospholipase C treatment of formaldehyde-blocked cream, a simple pH–mobility curve remained which could be attributed to the ionization of a carboxyl group with pK_a 4.7 suggesting the presence of either glutamate or aspartate on the surface of the membrane. Temperature variation in the bulk pK of anionogenic groups gives the heat of ionization for these groups as 1100 cal/mole which falls within the range for carboxyl groups.

Negative electrophoretic mobility could not be completely removed by diazomethane treatment indicating that complete esterification of such carboxyl groups could not be effected. However extended reaction times were not possible because of the instability of the cream in ethereal solution.

The evidence of protein amine and carboxyl groups and also of *N*-acetylneuraminic acid on the surface of the milk fat globule membrane suggests that the latter is largely composed of glycoprotein. This supports recent work in which sialoglycopep-

tides have been isolated from milk fat globule membrane following tryptic digestion[32]. The phosphate groups of phospholipids make a significant contribution to the surface charge density although the lack of effect of dodecyl sulphate on electrophoretic mobility within the range 10^{-6}–$5 \cdot 10^{-4}$ M indicates that there is little lipid in the outer surface of the membrane. Moreover the nature of the dependence of electrophoretic mobility both on dodecyl sulphate concentration within the range $5 \cdot 10^{-4}$–10^{-1} M and on ionic strength supports the finding that the surface of the milk fat globule membrane is primarily ionogenic.

After cream had been washed four times with distilled water subsequent washings produced little change in the pH–mobility curve. It would appear therefore, that ionogenic groups detected on the surface of our 9-times-washed cream cannot be ascribed to adsorbed milk serum components and are indeed an integral part of the membrane.

Sialoglycoproteins are known to be an important constituent of the outer surface of many mammalian membranes[33,34]. Our finding that milk fat globules have a surface of similar nature supports the contention that milk fat globule membrane has its origin in the plasmalemma of the mammalian alveolar cell.

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