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CROSSED IMMUNOELECTROPHORESIS OF BOVINE MILK FAT GLOBULE MEMBRANE PROTEIN SOLUBILIZED WITH NON-IONIC DETERGENT

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Summary

Detergent solubilized bovine milk fat globule membrane material studied by crossed immunoelectrophoresis combined with histochemical techniques revealed four major protein complexes. All four were found to bind to concanavalin A and three were identified as sialoglycoproteins. Xanthine oxidase activity was associated with the non-sialoglycoprotein precipitate. Immunoabsorption with intact milk fat globules showed an internal location of the xanthine oxidase, whereas the three other main proteins plus Mg^{2+}-ATPase and 5′-nucleotidase were disposed on the outer membrane surface. The major proteins from milk fat globule membrane and membrane material isolated from skim milk showed immunochemical identity.

Introduction

Extensive biochemical and morphological evidence implicates the milk fat globule membrane to be derived from the apical plasma membrane of lactating mammary cells [1—8]. Furthermore, membrane fragments bearing considerable resemblance with plasma membranes can be isolated from skim milk [9—11]. Both kinds of membrane material can be isolated with high yields and purity from milks of a variety of species [1] and since contamination with cytoplasmic organelles appears to be negligible at least in the bovine system, isolation of the membranes is very simple [12,13]. The mechanism of secretion of the milk fat globules [5,7,8] implicates a rather specialized function of the apical plasma membrane of the lactating mammary cells, and various lines of evidence [5,14,15] suggests it to be derived from intracellular membrane structures e.g. Golgi apparatus and rough endoplasmic reticulum. Although molecular rearrangements of the milk fat globule membrane may occur following secretion [6], it seems to be well qualified as a model system for investigations on biomembranes [1].

Crossed immunoelectrophoresis [16,17] has been found well suited for analysis of membrane proteins solubilized with non-ionic detergents [18—25] and recent modifications of this technique have made it possible to study a variety of the molecular characteristics of individual proteins without prior purification [16—19].

The aim of this report is to supplement the limited immunochemical work on the membrane material from milk [9,26,30] by establishing a reference pattern in crossed immunoelectrophoresis of the major membrane antigens and to perform a partial characterization of the individual components of this pattern.

Materials and Methods

Membrane preparation. Samples of uncooled bovine milk obtained 2—4 h after milking were separated in a cream separator (AlfaLaval, type C 36) and the cream was washed 3 times in 6 volumes of isotonic phosphate buffer, pH 6.8 at 30°C. After adjustment of the fat content to 33% (v/v) the cream was churned at 5°C. The pellet obtained by centrifugation of the buttermilk (in rotor SS-34, Sorwall RC 2-B) for 2 h at 39 000 × g_{av}, was suspended in 0.10 M glycine, 0.038 M Tris (pH 9.2, 5°C) and recentrifuged. This procedure was carried out twice, and the final pellet of fat globule membrane was stored at —20°C. In some experiments the supernatant after the first centrifugation of the buttermilk was salted out at 5°C in 3 M $(NH_4)_2SO_4$.

Membrane fragments from skim milk [10,11] were prepared according to ref. 10.

Antibodies. Rabbit antibodies against bovine whey proteins, bovine serum albumin and horse ferritin were obtained from Dakopatts A/S, Copenhagen. Rabbit antibodies against bovine milk fat globule membranes and bovine erythrocyte membranes were produced and purified to an immunoglobulin concentration of 5.5 mg/ml as previously described [18,31]. Traces of plasmin in the immunoglobulin preparations were inactivated by addition of 1000 K.I.E.*/ml aprotinin (Novo, Mainz).

Detergents and other reagents. The following detergents were used. Sodium dodecyl sulphate (BDH, London), Berol EMU-043 (a polyoxy-ethylene alcohol, MoDoKemi, Stennungsund, Sweden), Triton X-100 (*p-t-* octyl phenylpolyoxyethylene 9-10, Rohm and Haas, Philadelphia), and sodium deoxycholate (Merck, Darmstadt).

Bovine albumin (diagnostic reagent, Armours, Eastburne) and horse ferritin (Dakopatts A/S, Copenhagen) were used as marker antigens.

Protein assay. Protein was determined according to Lowry et al. [32] using the modification described in ref. 33.

Solubilization. Milk fat globule membranes were suspended at a protein concentration of 4 g/l in 0.010 M glycine, 0.0038 M Tris (pH 9.2, 5°C) containing 1% (w/v) Berol. After ultrasonic treatment at 20 000 cycles per second for 4 × 15 s on ice (Branson sonifier B 12 with microtip, Donburg, Conn.), the sample was centrifuged at 39 000 × g_{av} for 2 h at 5°C. The supernatant containing solubilized membrane proteins was used directly for further experiments or stored frozen at —20°C. A similar procedure was followed during solubilization

* K.I.E., kallikrein inhibitor einheiten.

with the other types of detergent. In some experiments proteins were solubilized directly from the surface of washed milk fat globules. Cream adjusted to a fat content of 50% (v/v) was frozen at −20°C. After thawing, Berol was added to a final concentration of 2% (w/v) and the mixture was incubated at 37°C for 1 h. The water phase containing the solubilized membrane proteins was recovered by centrifugation at 39 000 × g_{av} for 2 h.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. [34] using the slab apparatus described in ref. 35. All samples were made 3% with sodium dodecyl sulphate and 200 mM with dithiothreitol (Clelands reagent. Calbiochem) before analysis.

Immunoelectrophoretic methods. Rocket, crossed, tandem-crossed and crossed immunoelectrophoresis with an intermediate gel [16] was carried out in 1% agarose gels (batch 091A, Litex, Glostrup, Denmark) containing 0.050 M glycine, 0.019 M Tris, 0.002 M calcium lactate (pH 8.9, 16°C) and 1% (w/v) Berol as described in refs. 18 and 19. Normally 50 μg of Berol-solubilized milk fat globule membrane proteins was analysed. Crossed immunoelectrophoresis with Concanavalin A (Pharmacia Fine Chemicals, Uppsala) in the first dimension agarose gel (150 μg per cm^2 gel) was performed according to ref. 36.

Enzymatic degradation. Isolated membranes or Berol-solubilized membrane material was incubated for 8 h at 37°C (pH 8.7) with pronase (5 mg ml^{-1}) (B-grade, Calbiochem) and at pH 4.0 with pepsin (5 mg ml^{-1}) (2500 units/mg, Worthington). After solubilization, when necessary, the samples were used directly in immunoelectrophoresis and compared to untreated controls. Neuraminidase treatment of Berol-solubilized membrane material was carried out as described in ref. 19 using 250 units/ml of neuraminidase (V. Comma, Behring Werke, Marburg/Lahn).

Staining for enzymatic activity. Xanthine oxidase activity was demonstrated by the appearance of a purple-coloured precipitate in the immunoplates following incubation of the washed plates for 15 min at 37°C in 0.05 M Tris · HCl (pH 7.5) containing 0.02 M xanthine and 0.003 M 2,3,5-triphenyltetrazolium chloride. ATPase activity in the immunoplates was demonstrated by the technique of Gomori [37]. The washed immunoplate was incubated for 1 h at 37°C in 0.05 M Tris · HCl (pH 7.5) containing 0.010 M KCl, 0.040 M NaCl, 0.002 M $Pb(NO_3)_2$, 0.002 M Tris/ATP and either 0.005 M $CaCl_2$ or 0.005 M $MgCl_2$. The plate was then rinsed in water and soaked in 0.4 M $(NH_4)_2S$. Brown precipitates appeared in the gel corresponding to the sites of PbS formation. 5′-Nucleotidase activity was demonstrated by the same method using 0.005 M AMP instead of ATP. Control plates showed no staining when incubated in the absence of ATP and AMP. Analysis for acid and alkaline phosphatase activity was performed as described in ref. 38 with 0.005 M Naphthol-AS *-phosphate and 0.002 M 5-chloro-*o*-toluidine (Fast Red TR) as coupling salt. Esterase activity was assayed according to ref. 39 with 0.005 M α-naphthyl acetate and 0.002 M Fast Red TR. (All reagents from Sigma).

Phosphorylation of membrane proteins was demonstrated using radioactive phosphate according to Guthrow et al. [40]. An 0.5 ml aliquot of freshly prepared milk fat globule membranes (2 mg of protein) suspended in 0.05 M Tris ·

* AS, anilide substrate.

HCl (pH 8.0), 0.005 M $CaCl_2$, 0.010 M KCl and 0.040 M NaCl was mixed with 2 mol of [γ-^{32}P]ATP (20 μCi, NEN). After incubation for 10 min at room temperature the mixture was cooled on ice and the membranes were washed twice by centrifugation in 2 ml of the Tris · HCl buffer at 30 000 × g_{av} for 30 min at 5°C. The final pellet was solubilized in 1% (w/v) Berol and used for immunoelectrophoresis. After washing and drying of the immunoplate autoradiography was performed (4 days of exposure, RPX-omat film, Kodak).

Analysis for membrane sidedness [41,43]. In the immunoabsorption procedure, cream of fresh uncooled milk was gently prepared by centrifugation for 10 min at 2000 × g followed by two washings in 15 volumes of isotonic phosphate buffer pH 6.8 and adjustment of the fat content to 40% (v/v). The temperature of the cream was kept between 25 and 30°C during the whole procedure. Aliquots of 0.9 ml of anti-milk fat globule membrane and 0.1 ml of anti-horse ferritin (as a marker antibody) were mixed with 2.0, 4.0 and 8.0 ml of the washed cream, and sufficient buffer was added to give a final volume of 10 ml. Two series of experiments were carried out, one at 30°C and another at 5°C. After incubation for 1 h, the fat globules were removed by centrifugation at 2000 × g for 10 min and the infranatants were filtered through an 0.22 μm Millipore filter. The dilution of the antibodies in the infranatants was determined by rocket immunoelectrophoresis of horse ferritin. On basis of these data the volumes of antibody preparations from the different experiments were adjusted to contain identical titers against horse ferritin before use in crossed immunoelectrophoresis of milk fat globule membrane proteins.

Peroxidase catalysed ^{125}I-iodination [42] of milk fat globules was performed by mixing 5 ml of freshly prepared cream isolated at 30°C as described above with 50 μl lactoperoxidase from milk (57 units/mg, Sigma) and 5 aliquots of 5 μl H_2O_2 (0.1 μg/ml) added with a 1 min interval at 25°C. Another cream sample subjected to one cycle of freeze thawing was iodinated in a similar way. Iodination was stopped by the addition of 8 volumes of human serum albumin (10 mg/ml) and the cream was washed twice in 40 volumes of isotonic phosphate buffer pH 6.8 followed by a wash in 0.01 M glycine, 0.0038 M Tris pH 8.6, 25°C. The membrane proteins were solubilized directly from the cream as described in the section, solubilization.

Results

Before establishing a reference pattern in crossed immunoelectrophoresis of milk fat globule membrane proteins isolated from the buttermilk, the solubilizing effect of various detergents on these proteins was investigated. In Tris/glycine buffer the optimum conditions for solubilization using the non-ionic detergent Berol EMU-043 was obtained at an alkaline pH (9.2) and a low ionic strength (0.01) at a detergent/protein ratio of (3 : 1, w/w). Under these conditions 20—30% of the total protein remained in the supernatant after centrifugation at 5.8 × 10^6 g · min. Similar figures were obtained for Triton X-100 and Lubrol WX and the ionic detergent sodium deoxycholate, whereas sodium dodecyl sulphate under the same conditions solubilized 80—90% of the total protein at 15°C. Repeated extractions of pelleted membrane protein with Berol EMU-042 showed that 80% of the total amount of solubilized protein was extracted during the first cycle.

The use of non-ionic detergent as a solubilizing agent has proved its value in the immunoelectrophoretic analysis of amphiphilic membrane proteins [18,19,28], whereas proteins treated with sodium dodecyl sulphate are known to give bizarre patterns in crossed immunoelectrophoresis [28]. For the immunochemical analysis it was of importance to establish whether the difference in solubilizing effect between the non-ionic detergent and sodium dodecyl sulphate was of a qualitative or merely a quantitative nature. This was examined by comparing the patterns obtained in sodium dodecyl sulphate-polyacrylamide gel electrophoresis of milk fat globule membrane proteins solubilized in Berol EMU-043, Triton X-100, sodium deoxycholate and sodium dodecyl sulphate (Fig. 1). With the exception of a relative abundance of protein in 2 diffuse bands in the low molecular weight area in the samples solubilized in sodium deoxycholate and sodium dodecyl-sulphate, the patterns are identical showing five major bands. This finding was confirmed by crossed immunoelectrophoresis in Berol-containing gels of the individual samples all showing the same basic precipitation pattern regardless of the type of detergent used [28]. Berol EMU-043 was then chosen for further studies.

Crossed immunoelectrophoresis of membrane proteins

The reference pattern obtained in crossed immunoelectrophoresis of Berol-solubilized bovine milk fat globule membrane proteins (Fig. 2) reveals four main precipitates (numbered 1—4) and a few weak precipitates (arrows). The four main precipitates were readily identifiable in crossed immunoelectrophoresis of membrane proteins from each of eleven successive milk preparations, whereas the weak precipitates appearing with less constancy were not considered further. Membrane proteins solubilized with Triton X-100 showed the same pattern. Pronase and pepsin treatment of the membrane proteins prior to immunoelectrophoresis changed the morphology of all precipitates, thus revealing all antigens to contain polypeptides.

Considerable differences in the position and appearance of the four main precipitates were observed from one preparation to another. Antigen 2 and 4 showed the greatest heterogeneity in electrophoretic migration velocities, both spanning the entire γ- and β-mobility area at pH 8.9, giving rise to broad and non-symmetric precipitates. These precipitates were sometimes seen to have a double contour (Figs. 6A, and 7B) and to split into several fine branches at their anodal ends (Fig. 4). This either indicates that they represent protein complexes or have been subjected to proteolytic degradation [43]. Antigen 1 migrated with α-mobility. The corresponding precipitate appeared bell shaped and distinct. Antigen 1 was consistently found in relatively high concentrations in the supernatant after centrifugation of the buttermilk. Likewise, it was the only milk fat globule membrane protein readily demonstrable in crossed immunoelectrophoresis of untreated skim milk preparations [29]. This finding suggests a rather weak binding to the milk fat globule membrane or a specific cleavage mechanism of this protein.

Removal of the detergent from the gels drastically changed the precipitation pattern obtained in crossed immunoelectrophoresis. The migration velocities of antigen 1,2, and 3 increased, and at the same time the area below all the precipitates increased. Furthermore, precipitate 2 and 4 showed several peaks and

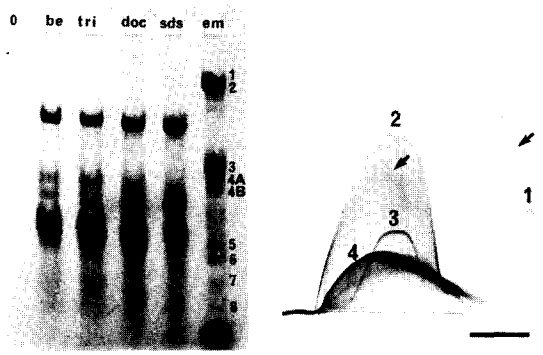

Fig. 1. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis of 25 μl of bovine milk fat globule membrane proteins solubilized with different detergents. From left to right: In buffer without detergent (○), with 1% Berol EMU-043 (be), with 1% Triton X-100 (tri), with 1% sodium deoxycholate (doc), and with 1% sodium dodecyl sulphate (sds). To the right 50 μg sodium dodecyl sulphate-solubilized human erythrocyte membrane proteins (em) has been included as a molecular weight reference with major bands labelled according to ref. 34. All samples were treated with 3% sodium dodecyl sulphate and 200 mM dithiothreitol prior to electrophoresis in 5% acrylamide with 2.5% crosslinking. Staining: Coomassie Brilliant Blue R.

Fig. 2. Crossed immunoelectrophoresis of 50 μg (25 μg) Berol-solubilized bovine milk fat globule membrane proteins in agarose gel containing 1% (w/v) Berol EMU-0.43 and 0.7 $\mu l \cdot cm^{-2}$ of anti-bovine milk fat globule membrane antibodies. First dimension electrophoresis: 10 $V \cdot cm^{-1}$ for 60 min (anode to the right); second dimension: 3 $V \cdot cm^{-1}$ for 18 h (anode at the top). The four major precipitates are indicated with numbers and the irregularly appearing precipitates with arrows. Staining: Coomassie Brilliant Blue R. The bar represents 1 cm.

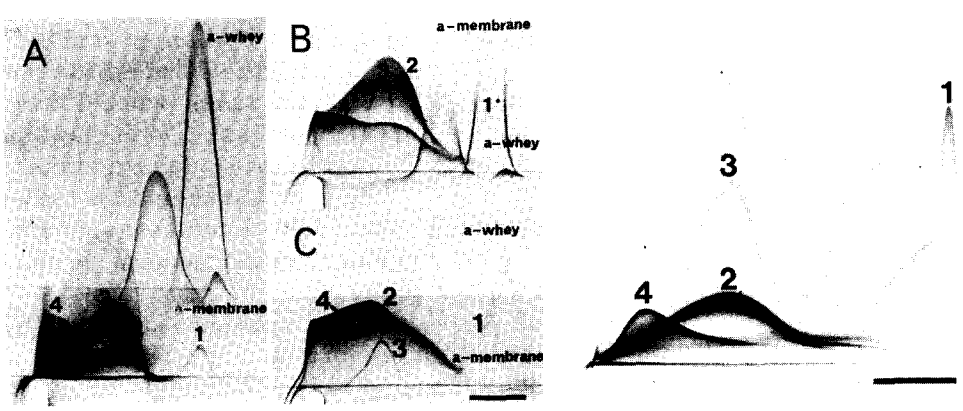

Fig. 3. Examination for membrane specificity. Crossed immunoelectrophoresis with an intermediate gel of (A) 50 μg (25 μl) Berol-solubilized milk fat globule membrane proteins contaminated with whey proteins run against anti-milk fat globule membrane antibodies in the intermediate gel (3.5 $\mu l \cdot cm^{-2}$) and anti-bovine whey antibodies in the upper gel (5.3 $\mu l \cdot cm^{-2}$). (B) an identical sample run against anti-bovine whey antibodies in the intermediate gel (27 $\mu l \cdot cm^{-2}$) and anti-milk fat globule membrane antibodies in the upper gel (0.7 $\mu l \cdot cm^{-2}$). (C) 50 μg (25 μl) Berol-solubilized milk fat globule membrane proteins from washed pellet run against the same antibody set up as in (A). The bar represents 1 cm.

Fig. 4. Crossed immunoelectrophoresis of 30 μg (15 μl) Berol-solubilized bovine skim milk membranes. Conditions otherwise as in Fig. 2. The bar represents 1 cm.

precipitate 4 became blurred. These reactions typical of detergent-solubilized amphiphilic membrane proteins [20] indicate molecular rearrangements of the proteins upon removal of the detergent.

Reaggregation of the detergent-solubilized membrane proteins seen as an unspecific precipitation in the application well and by increased turbidity of the samples took place during prolonged storage at −20° C, but were of no significant importance in samples stored for periods of less than one month at this temperature.

The specific association of antigen 1—4 with the milk fat globules was demonstrated by crossed immunoelectrophoresis with an intermediate gel containing either antibodies against bovine whey proteins or against bovine milk fat globule membranes (Fig. 3). The antigen used in Figs. 3A and B was obtained by solubilization in Berol of membrane proteins from intact globules in thrice washed cream. This antigen preparation being contaminated with small amounts of whey protein was then compared with membrane material prepared by the standard method. Antibodies against milk fat globule membrane proteins (in the intermediate gel of Fig, 3A) showed little activity against bovine whey proteins as seen from the presence of precipitates in the upper gel of Fig. 3A containing antibodies against bovine whey proteins. However, inversion of the antibody-containing gels (Fig. 3B) showed that the antibodies against bovine whey were active also against the milk fat globule membrane proteins. The washings of the buttermilk pellet prior to solubilization in Berol removed most of the contaminating whey proteins as judged from the disappearance of the whey-precipitates in the upper gel of Fig. 3C with the same antibody set up as in Fig. 3A. This indicates that the milk fat globule membrane proteins are sharply defined against the bulk protein of the whey.

Comparison of membrane proteins from different milk phases

Berol-solubilized skim milk membranes [10] analysed by crossed immunoelectrophoresis (Fig. 4) gave rise to a precipitation pattern similar to the one obtained with Berol-solubilized milk fat globule membranes pelleted from buttermilk of the same milk batch. The immunochemical identity of the four membrane proteins from the two sources was established by tandem-crossed immunoelectrophoresis [16]. The skim milk membranes contained a higher proportion of antigen 1 and 3, and in this plate (Fig. 4) the corresponding precipitates are fusing i.e. revealing a reaction of immunochemical identity [16].

Crossed immunoelectrophoresis of Berol-solubilized proteins obtained by salting out the supernatant after centrifugation of the buttermilk likewise revealed a pattern similar to the one obtained with the corresponding pellet. Furthermore, the proteins captured in the butter during churning were not found to be antigenically different from those released to the buttermilk. Milk fat globule membrane proteins thus appear to be distributed throughout the milk phases, although in unprocessed milk the largest amount is found tightly bound to the surface of the milk fat globules.

Concanavalin A-binding membrane proteins

Incorporation of free concanavalin A in the first dimension gel in crossed

immunoelectrophoresis constitutes a simple means for studying interactions between this lectin and appropriate receptor-molecules [36], even in the presence of non-ionic detergent [18,22,30]. A crossed immunoelectrophoresis of milk fat globule membrane proteins performed with free concanavalin A incorporated into the first dimension gel is shown in Fig. 5A. When compared to a control without concanavalin A (Fig. 5B) all the four major antigens are seen to interact with the lectin. Artificially introduced bovine serum albumin is not affected by the presence of concanavalin A. Note the formation of an affinity precipitate [36] (open arrow) in the first dimension gel of Fig. 5A. The observed interactions were inhibited by the addition of α-methyl-D-glycopyranoside (1% w/v) to the gel [30] indicating the specificity of the reaction. On the basis of these experiments, we concluded that all four major antigens are glycoproteins containing carbohydrate parts with glucose and/or mannose residues [53].

Analysis for sialoglycoproteins

The negative charge of sialic acid groups contribute significantly to the mobility of sialoglycoproteins during electrophoreses at pH 8.9 [18,19,22,44]. In crossed immunoelectrophoresis of neuraminidase-treated milk fat globule membrane proteins a significant decrease in migration velocity was observed for antigens 1, 2, and 3 as compared to an untreated control, indicating their nature as sialoglycoproteins.

Enzymatic activity of the immunoprecipitates.

The presence of several enzymatic activities of the immunoprecipitates was demonstrated by histochemical methods. ATPase activity was associated with a precipitate that only became visible during the enzymatic staining procedure (Fig. 6B). The anodal part of this precipitate was in some plates seen to fuse in a reaction of partial identity with precipitate 2 (cf. Fig. 6A) which is the same plate after staining with Coomassie Brilliant Blue R. The ATPase required the

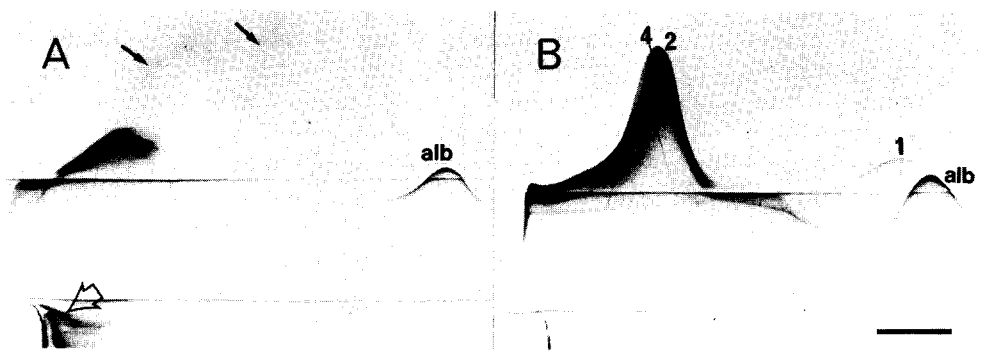

Fig. 5. Crossed immuno-affinoelectrophoresis of 50 μg (25 μl) of Berol-solubilized bovine milk fat globule membrane material (A) with free concanavalin A in the first dimension gel (150 μg · cm^{-2}) and (B) a control without concanavalin A. A blank intermediate gel is inserted between the antibody containing gel and the first dimension gel. (alb) denotes bovine serum albumin precipitate. Open arrows indicates affinity precipitate and solid arrows two new precipitates not seen in (B), probably the result of a coprecipitation phenomenon with concanavalin A [36]. Except for the addition of anti-bovine serum albumin to the second dimension gel conditions are as in Fig. 2. The bar represents 1 cm.

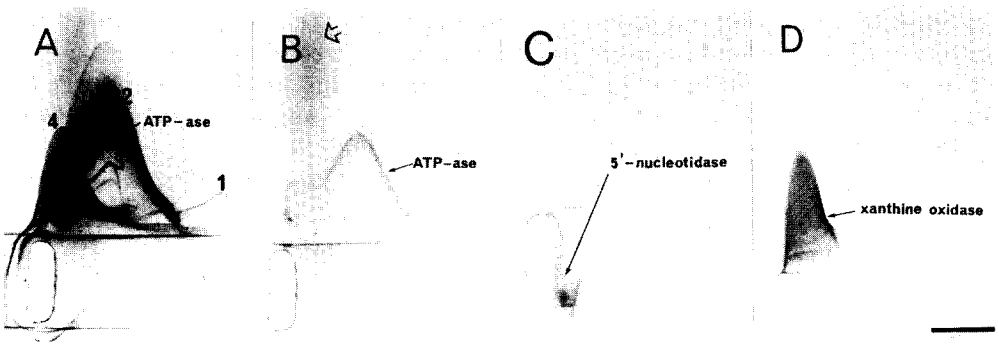

Fig. 6 Crossed immunoelectrophoresis of 50 μg (25 μl) of Berol-solubilized bovine milk fat globule membrane material. (A) stained for ATPase activity and then for protein with Coomassie Brilliant Blue R. (B) same plate before protein staining. (C) stained for 5′-nucleotidase activity and (D) stained for xanthine oxidase activity. For staining conditions see Materials and Methods. Conditions otherwise as in Fig. 2. The bar represents 1 cm.

presence of divalent cation for its activity, the reaction being faster in the presence of 5 mM Ca^{2+} than in 5 mM Mg^{2+}. Neither Na^{+} or K^{+} was necessary for the reaction to take place, and accordingly no inhibitory effect could be demonstrated by the presence of ouabain.

The 5′-nucleotidase activity was located near to the application well (Fig. 6C) in a position different from any of the four major precipitates. In some plates however, a faint simultaneous staining of precipitate 2 was seen, but in a pattern different from the one obtained using ATP as a substrate. As for the ATPase the staining was faster and became more intense when Mg^{2+} was replaced by Ca^{2+} during the incubation with substrate.

Alkaline phosphatase was demonstrated as a very diffuse precipitate above the application well. In some plates stained for ATPase activity a diffuse staining reaction took place in a location corresponding to this precipitate (open arrow Fig. 6B). Neither acid phosphatase activity nor esterase activity were demonstrable in the immunoplates.

Precipitate 4 was found to exhibit the xanthine oxidase activity (Fig. 6D). The staining reaction of this enzyme was completed within 30 min using xanthine as a substrate, whereas only a faint staining was seen after incubation for 24 h using xanthosine as a substrate under the same conditions.

The enzyme activities decreased upon storage of the solubilized membrane material at −20°C. Thus, after one week only xanthine oxidase activity was readily demonstrable.

Analysis for formation of phosphorylated membrane proteins revealed a single radioactive precipitate whose location in the plate was identical to that of the ATPase precipitate. Also in this system Ca^{2+} seemed to be superior to Mg^{2+} as a divalent cation during the incubation as judged from the intensity of blackening of the films.

Topographical distribution of the membrane proteins

Two methods, immunoabsorption [23,42] and peroxidase catalysed ^{125}I-iodination [42], were employed in order to detect a possible sidedness in the

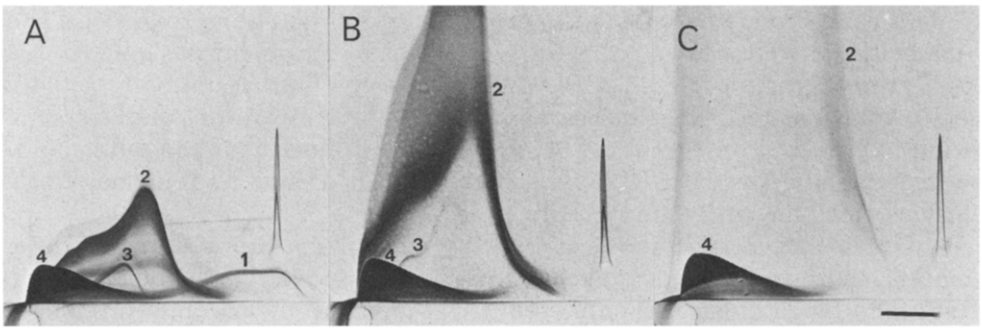

Fig. 7. Crossed immunoelectrophoresis of 75 μg (40 μl) of Berol-solubilized bovine milk fate globule membrane material run against (A) unabsorbed anti-milk fat globule membrane antibodies (1.0 $\mu l \cdot cm^{-2}$), (B) the same antibodies absorbed with 2.2 ml of 40% (v/v) washed cream per ml of the antibody preparation and (C) absorbed with 8.9 ml of 40% (v/v) per ml of the antibody preparation. The uniform dilution of the antibodies is proved by the uniform heights of the rocket precipitates to the right presenting horse ferritin (1.0 μg horse ferritin run against 0.11 $\mu l \cdot cm^{-2}$ of anti-horse ferritin). The plates have been stained for protein and xanthine oxidase activity. The bar represents 1 cm.

distribution of the individual antigens across the milk fat globule membrane. Crossed immunoelectrophoresis of Berol-solubilized milk fat globule membrane proteins using two antibody samples absorbed with increasing amounts of intact fat globules is shown in Figs. 7B and C. When compared to a control containing non-adsorbed antibodies (Fig. 7A) it is clearly seen from the successive increase in area of precipitates 1, 2, and 3, that antibodies against these antigens are being removed during the absorption procedure, whereas the antibody titer against antigen 4 remains virtually unchanged. When these plates were stained for xanthine oxidase activity, this was still found to be exhibited by precipitate 4, thus suggesting this enzyme to be located in the interior of the membrane. In

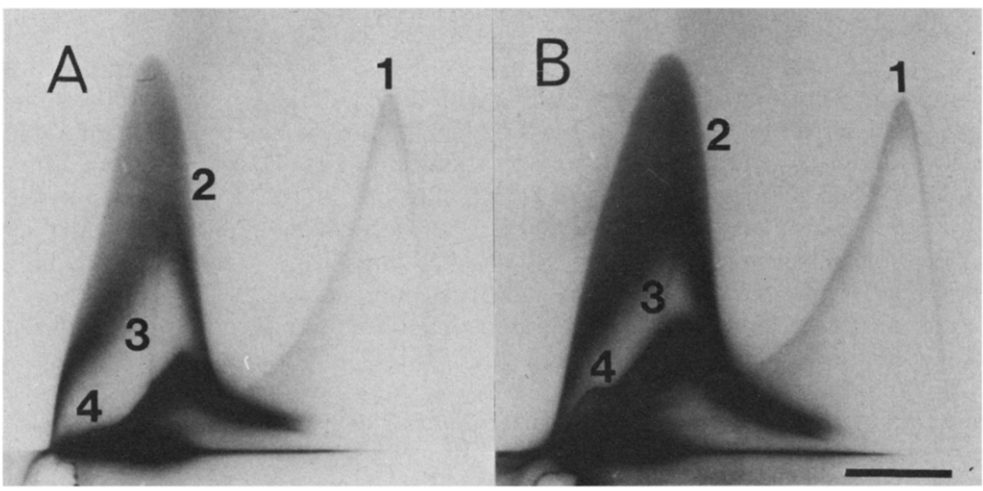

Fig. 8. Autoradiograph of a crossed immunoelectrophoresis of Berol-solubilized bovine milk fat globule membrane material obtained after lactoperoxidase catalysed ^{125}I-iodination of (A) intact milk fat globules and (B) milk fat globules subjected to one cycle of freeze-thawing. The plates have been exposed for 2 days. Conditions otherwise as in Fig. 2. The bar represents 1 cm.

an identical set of experiments the precipitates corresponding to ATPase and 5′-nucleotidase were shown to increase their height in a manner similar to the one seen for proteins 1, 2, and 3, thus indicating a loss of antibody activity against both these enzymes during absorption. The temperature of the system during the absorption procedure was found to be crucial for the outcome of the experiment. Thus, at 5°C the antibody activity against all four major proteins decreased markedly during absorption.

Peroxidase-catalysed ^{125}I-iodination [42] of proteins exposed on the outer membrane surface of intact milk fat globules was used as the second approach. Intact milk fat globules were subjected to ^{125}I-labelling under conditions identical to the ones used for the immunoabsorption procedure. Two samples prepared from the same cream batch at two different temperatures were compared. The autoradiographs of the crossed immunoelectrophoresis plates of each of the two membrane preparations are shown in Fig. 8. In disagreement with the results obtained in the immunoabsorption experiments, all four major antigens were labelled by this procedure, thus indicating partial exposure of protein 4 on the surface of at least a fraction of the milk fat globules.

Discussion

Solubilization of the major milk fat globule membrane proteins with non-ionic detergents was found to be incomplete but qualitatively non-selective. This taken together with the irregular morphology of the resulting precipitates in crossed immunoelectrophoresis suggests a partial and heterogenous dissociation of the membrane protein complexes upon treatment with these agents.

The lower efficiency of non-ionic detergents as dissociating agents as compared to sodium dodecyl sulphate [52] might also account for the fact that only four major antigens were demonstrated in crossed immunoelectrophoresis, whereas at least five major polypeptide chains were revealed by dodecyl sulphate-polyacrylamide gel electrophoresis of the same samples (cf. Fig. 1 and refs. 45 and 46)). Thus, some of the four major antigens are presumably containing more than one polypeptide chain not dissociable by non-ionic detergents. This idea is substantiated by the electrophoretic heterogeneity of some major antigens, notably 2 and 4, and by the finding of ATPase activity in a precipitate showing varying degrees of fusion with precipitate 2. Furthermore, the xanthine oxidase activity has been shown not to be released as the free enzyme during solubilization with Triton X-100 [47], suggesting precipitate 4 to be a complex between xanthine oxidase and other polypeptides.

The antigenic similarity of fat globule membranes and membrane material from skim milk suggests a common origin of these membranes.

The binding of all four main proteins to concanavalin A has earlier been demonstrated in isotachophoresis experiments [30], and is in agreement with the results of Keenan et al. [48].

All the sialic acid of the milk fat globule membrane has been shown to be accessible to neuraminidase treatment of the outer surface of the membrane [49,50]. It is unknown whether this finding correlates to an actual sidedness of the membrane or reflects a disruption of the membrane structure. The result of

this study indicates that little or no sialic acid was associated with protein 4, whereas proteins 1, 2, and 3 all were shown to be sialoglycoproteins. Furthermore protein 4, carrying the xanthine oxidase activity was found to be at least partially hidden from the outer surface of the milk fat globule as judged from immunoabsorption experiments, whereas proteins, 1, 2, 3, ATPase and 5′-nucleotidase all were exposed on the outer surface. Such an internal localization of protein 4 would make it less likely to be released in large amounts to the skim milk phase during shedding of membrane material from either plasma membrane or fat globule membrane as compared to the other protein components of the system. In agreement with this is the finding by Kitchen [10] of a higher specific activity of 5′-nucleotidase and Mg^{2+}-ATPase in the skim milk membranes than in the milk fat globule membranes, but a reverse relationship for the xanthine oxidase activity. The exposure of antigenic determinants of the xanthine oxidase carrying protein upon cooling of the fat globules may be a result of crystallisation of the triglyceride core of the globules disturbing the membrane structure. Morphological changes of the membrane together with a release of xanthine oxidase activity to the milk serum have previously been shown to occur upon cooling of the fat globules [1,3]. These observations speak in favour of an asymmetry in distribution of the protein constituents in the uncooled membrane and at the same time argue against intensive shedding of the membrane material in the hour immediately following secretion.

The disposition on the outer membrane surface of antigenic determinants corresponding to 5′-nucleotidase and Mg^{2+}-ATPase is in agreement with studies comparing the activities of these enzymes before and after release from the milk fat globules [4]. Due to low antibody avidity against the alkaline phosphatase we were unable to determine the relation of this enzyme to the membrane surfaces.

All four major antigens were found to be accessible to peroxidase catalysed ^{125}I-iodination of intact (uncooled) milk fat globules. This is in contrast to the hiding of the antigenic determinants of protein 4 deduced from the immunoabsorption experiments. The divergence might be explained by a difference in sensitivity of the two experimental approaches. Thus, ruptures in the membranes of even a small percentage of the milk fat globules could result in detectable ^{125}I-iodination of protein 4 [50] and at the same time reveal too little antigenic determinants of this protein to remove significant amounts of the corresponding antibodies in the immunoabsorption procedure. Also, hiding of the antigenic determinants of protein 4 in intact membranes does not preclude disposal of antigenically inert tyrosine-containing parts of this molecule on the outer membrane surface. An analogous behaviour has recently been demonstrated for the major intrinsic protein of human erythrocyte membranes known to spann the membrane [19]

The basic immunochemical characterization of the milk fat globule membrane presented in this study underlines the applicability of the crossed immunoelectrophoresis in investigations on membrane proteins. In future applications, the immunoelectrophoretic methods will be used to monitor separation of the membrane components on a preparative scale and to further elucidate their role in maintenance of the membrane structure. Preliminary investigations along these lines have been published elsewhere [30].

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