CHAPTER II

REVIEW OF LITERATURE
Milk fat globule membrane (MFGM) envelopes the fat globules in milk and thus helps to stabilise the fat emulsion. In 1974, Van Leeuwenhoek observed the fat globules in milk with the aid of a microscope. Various theories were put forward in 19th century regarding the ability of MFGM to stabilise the fat emulsion (Anderson, 1840; Babcock, 1889 and Storch, 1897).

Milk fat globules, free from milk plasma proteins, were first isolated by Hattori (1928). Palmer and Samuelsson (1934) observed phospholipids as components of MFGM. They suggested that MFGM exists as a lipoprotein complex and phospholipids help in emulsifying the fat globules. Studies on MFGM by Titus et al (1928) led to the hypothesis that MFGM consisted of milk plasma proteins adsorbed on to the fat globules. But Herald and Brunner (1967) showed that the physical-chemical properties of MFGM proteins are distinctly different from the milk plasma proteins.

Koeman et al (1970) observed that the phospholipids and proteins of MFGM are identical with those of plasma membrane of the lactating mammary cell. The molecular weights of bovine MFGM proteins have been determined by various workers (Kobylika and Carraway, 1973;
Anderson et al., 1972a; Anderson et al., 1974; Mangino and Brunner, 1975). High molecular weight proteins and glycoproteins which are characteristic components of a plasma membrane (Guidotti, 1972), are components of MFOM.

Ballie and Morton (1958) reported that some enzymatic activities which they detected in cream were of microsomal origin. Many enzymes which are usually associated with plasma membrane have been detected in MFOM (Dovben et al., 1967; Patton and Trams, 1971).

Huang and Keenan (1972a) studied the properties of 5'-nucleotidase, a marker enzyme used for plasma membrane, from bovine MFOM. Kinetic studies showed a close resemblance of this enzyme with 5'-nucleotidases of plasma membrane origin (Song and Bedansky, 1967; Widnell and Onkeless, 1968). The properties of ATPase of MFOM (Huang and Keenan, 1972b) resembled the properties of the enzymes present in several other tissues (Skeu, 1965; Emaneot and Bos, 1966; 1968; Parkinson and Radde, 1971).

MFOM has been shown to be a true biological membrane by extensive electron microscopic studies (Bargmann and Kneep, 1959; Bargmann et al., 1961). The above studies led to the hypothesis that MFOM is derived from plasma membrane of lactating mammary cell. Wooding (1971a; 1971b) suggested that Golgi vesicles are also involved in the formation of MFOM. MFOM has been shown undergoing structural changes after milk secretion.
(Keenan et al., 1971). Keenan et al. (1970; 1971) and Bauer (1972) also supported the above observation by morphological studies.

All the above morphological and biochemical studies on MFPM support the hypothesis that the membrane is derived primarily from the plasma membrane of the lactating cell. Hence, MFPM has been suggested a promising system for the study of the organisation of the components of plasma membrane of mammary cell (Patton and Trams, 1971) and for the study of protein-protein as well as protein-lipid interactions in a membrane (Kobylika and Carraway, 1972).

**Procedures for isolation of MFPM.**

To characterise any membrane, the membrane under study should be isolated free from the contaminants that might have adsorbed on to the membrane. The isolation of MFPM is rather simple unlike the isolation of internal membranes of a cell. All that need be done is to separate fat globules from milk and to strip the membrane from the fat globules. Because MFPM can easily be isolated, this membrane is among the most widely studied of all membranes (Keenan et al., 1974).

The isolation procedure of MFPM was first developed by Palmer and coworkers (Palmer and Samuelsson, 1934; Rimpila and Palmer, 1935; Jonness and Palmer, 1945). Their procedure for isolation of MFPM was based on
dilution and washing of cream. They extracted the lipid materials from the proteinaceous materials of MFGM by refluxing the isoelectric, dialysed MFGM with successive portions of acetone, ethanol and diethyl ether. Herald and Brunner (1957) isolated MFGM materials by a more mild method to minimize the protein denaturation during the course of isolation. Their procedure involved washing the cream six times with distilled water to remove the adsorbed milk plasma constituents. The washed fat globules were churned to release the MFGM material which was concentrated by ammonium sulphate precipitation. Little et al. (1960b) have reported that 85% activities of alkaline phosphomonoesterase and xanthine oxidase in cream were lost during washing four times with distilled water. Swep et al. (1968) observed that to preserve the organization of the membrane structure during isolation, the washing operation should be restricted to the minimum number of three by which all the milk plasma proteins would be removed. They also found that the yield of the membrane increased per gram fat when they used sucrose-saline solution for washing the fat globules. It has been observed that washing of the milk fat globules with sucrose solution resulted in the retention of more enzymatic activities associated with MFGM during the isolation of the membrane (Dawben et al., 1967).

Keeson et al. (1970) isolated MFGM by a freeze-
thawing procedure. Milk fat globules were recovered by centrifuging the milk and the fat globules were washed with 0.9% sodium chloride. The fat globules were subjected to freeze-thawing and the MF GM was isolated by centrifugation. The purity of the membrane material was determined by density gradient centrifugation. Bracey et al. (1972) avoided continuous creaming in a separator as well as washing and churning the fat globules. The cream obtained by centrifuging milk was adjusted to 30% fat, 11 g of sucrose was added per 100 ml of cream. The above cream was centrifuged at 44,000 x g for 3 hr to obtain the membrane material.

Kobylika and Carraway (1972) separated the cream from the chilled milk. The cream was washed with imidazole buffered sucrose solution at room temperature. The solid wet cream (33% fat) was subjected to homogenization or to freeze-thawing and the membrane material was isolated by centrifuging at 40,000 x g for 1 hr. The sediment obtained was again washed twice with imidazole buffered sucrose solution. They noted that a consistent membrane yield was obtained when the fat globules were washed with imidazole buffered sucrose solution rather than with sucrose or saline solution. The yield of membrane from cream obtained by this procedure ranged from 4 to 9%, based on protein recovery.

Thus it is evident that various workers have used different procedures for the isolation of MF GM. The method
of isolation of MFGM proteins developed by Herald and Brunner (1987) appears to be suitable for the study on the compositional aspects of MFGM proteins as it represents the total amount of proteins obtained from the membrane. But recently, Mangino and Brunner (1975) have shown by electrophoresis that the protein species originally present in the MFGM associated with high molecular species concomitant with removal of lipid moieties. So it appears that for electrophoretic characterisation of MFGM proteins, the studies should be conducted on MFGM itself and not on delipidated proteins. In the freeze-thawing procedure for isolation of MFGM, of Kobylka and Carraway (1972), the fraction sedimented at 40,000 x g from the membrane material represents MFGM. Although the chemical composition of this fraction reported by the above authors was different from other reported value for MFGM (Brunner, 1989), it has been shown by electrophoresis that this fraction contained all the major protein components present in the washed cream. Since this fraction represented the MFGM, at least with respect to protein components, it appears that the membrane prepared by the procedure of Kobylka and Carraway (1972) can be used for selective solubilisation studies of MFGM proteins.

Lipoprotein nature of MFGM

Earlier workers thought MFGM is proteinaeous in nature (Ascherson, 1840; Storch, 1897; Babcock, 1888;
Hatto ri, 1925; Titus et al, 1929. Palmer and co-workers (Palmer and Samuelsson, 1924; Wiese and Palmer, 1932; Palmer and Wiese, 1933; Wiese and Palmer, 1934) showed that phospholipids are also components of MFOM. Vitamin A was observed as a constituent of MFOM (White et al, 1954). Thompson et al (1961) identified carotenoids, cholesteryl esters, cholesterol, squalene, triglycerides, phospholipids and unesterified fatty acids in MFOM. Alexander and Lusena (1961) separated the deoxycholate dissociated lipoproteins into six different fractions, which differed in chemical composition, by differential centrifugation. Harwalkar and Brunner (1965) fractionated MFOM into low and high density lipoproteins. Sedimentation velocity patterns of the above lipoproteins were found to be different. Hayashi and Smith (1965) solubilised 45% of the membrane material by deoxycholate treatment and chemical analysis showed that lipids and proteins were present in equal amounts in the above preparation. Their findings led to the view that the membrane surrounding the fat globules consists of two types of lipidprotein complexes in equal amounts and distinguishable on their solubility in water. Chien and Richardson (1967a,b) demonstrated, without employing any dissociating agent, that the membrane material could be progressively eroded by agitation into five principal lipoprotein fractions differing significantly in gross composition. Swope and Brunner (1970) similarly
fractionated MFGM lipoproteins into three lipoprotein fractions, namely 7800, 2800 and 350. They contained 17.5%, 44.7% and 55.3% lipids, respectively.

Keenan et al. (1968) observed similarity between the fatty acid composition of phospholipids from MFGM and from plasma membrane of lactating mammary cell. The distribution of phospholipids in MFGM was found to be 20.6% sphingomyelin, 41.1% phosphatidyl choline, 28.2% phosphatidyl serine, 5.8% phosphatidyl inositol, and 20.7% phosphatidyl ethanolamine (Keenan et al., 1972a). Breshe et al. (1973) have characterised the polar and non-polar lipids of MFGM isolated from bovine and human milk.

Benerjee et al. (1974) separated buffalo MFGM into three fractions according to the procedure of Swepe and Brunner. They observed some differences in chemical composition of proteins of various lipoprotein fractions. Brunner et al. (1973) observed that electrophoretic patterns of delipidated proteins of these fractions were similar. Hence, they indicated that the proteins were uniformly distributed throughout the membrane structure and that centrifugal separation only reflected the degree of lipid mediated aggregation.

Thus it can be seen that the lipid components of MFGM have been studied and characterised. The lipoproteins of MFGM have been separated depending on density. Low density and high density lipoproteins have
been found to differ in their gross chemical composition. Banerjee et al. (1974) in a comparative study on the fat globule membrane proteins of buffalo and cow observed that the gross composition of membrane proteins of the two species are essentially similar. Both species were observed to contain 7% carbohydrate, indicating the presence of significant amounts of glycoproteins.

Chemical composition of MFPM proteins

Although Storch (1897) reported that MFPM contained carbohydrate, Wiese and Palmer (1934) were the first to demonstrate the difference in the composition between MFPM proteins and other milk proteins. They reported that it contained 13.22% nitrogen, 0.06% sulphur and 0.33% phosphorus. Herald and Brunner (1987) developed a new procedure for the isolation of MFPM proteins. They cleaved the lipoproteins with 38% ethanol in ether (0-8°C) and isolated the delipidated proteins which were further separated into two fractions based on their solubility in 0.02 M NaCl. The soluble whitish fraction contained 11.10% nitrogen while the insoluble reddish brown fraction contained 13.3% nitrogen. Thompson and Brunner (1989) separated the carbohydrates present in the soluble fraction by paper chromatography. They reported that this fraction contained 2.6% hexose, 1.08% hexosamine, 0.71% fucose and 2.0% sialic acid. It was also observed that the soluble fraction of delipidated
MPGM proteins contained 0.68% phosphorus and 2.68% to 4.04% hexose. A mucoprotein isolated from fat/plasma interface of cow’s milk was found to contain 5.2% hexose, 3.9% hexosamine, 4.6% sialic acid and 0.68% phosphorus (Jackson et al., 1962). Neuraminidase released 65% of the total sialic acid present in the mucoprotein within 6 hr. Mild acid hydrolysis (0.1 N HCl) at 37°C released almost all sialic acid in 72 hr. Chian and Richardson (1967b) separated MPGM into five lipoprotein fractions which differed in gross chemical composition with respect to protein and lipid. But they observed glucosamine (1.1% to 1.4%) and galactosamine (0.5% to 2.5%) were more or less equally distributed in all lipoprotein fractions. Swepe et al. (1968) separated the membrane lipoprotein complex by differential centrifugation into three principal fractions. Following the removal of lipid moieties by dissociating agents, the residual proteins were classified on the basis of their solubility in aqueous systems. They isolated a soluble glycoprotein component which contained 16 to 19% carbohydrates as estimated from analysis of hexose, hexosamine and sialic acid. Swepe and Brunner (1970) separated MPGM lipoproteins into three fractions, namely 7S, 23S and 30S. The carbohydrate content of the delipidated proteins of these fractions were 6.6%, 8.4% and 10.3%, respectively. Banerjee et al. (1974) separated the buffalo MPGM
according to the procedure of Swope and Brunner (1970) and observed 5.58%, 8.48% and 10.82% carbohydrate in delipidated proteins of 7S, 23S and 35S lipoprotein fractions, respectively. The above observations showed that the proteins of low density lipoproteins of both cow and buffalo MFGM contained higher amount of carbohydrates than the corresponding high density lipoproteins.

Milder and Koppejan (1983) observed that copper and iron were associated to a great extent with the MFGM. Herald et al. (1987) estimated calcium, magnesium, copper, iron, manganese, molybdenum, silver, zinc and phosphorus in MFGM. They noted that MFGM contained mineral elements in higher concentration than reported for whole milk. They suggested that these trace elements on MFGM may be constituent elements of various enzymes on metabolic processes. Ramachandran and Whitney (1960) isolated a membrane fraction rich in copper, but low in iron and molybdenum which had high alkaline phosphatase activity. 10 to 15% of copper associated with MFGM could be easily washed off (Samuelsson, 1986). Copper was found to be evenly distributed in various lipoprotein fractions of MFGM while iron was found to be concentrated in high density lipoproteins (Richardson and Cuss, 1965; Chien and Richardson, 1987b).

Amino acid composition of bovine MFGM has been studied (Hare et al., 1982; Brunner et al., 1983a). Herald and Brunner (1987) published amino acid composition of
soluble and insoluble membrane proteins. Amino acid composition of five lipoprotein fractions of MFGM was found to be similar (Chien and Richardson, 1967a,b). Kobylka and Carraway (1972) observed similarity in amino acid composition of bovine MFGM proteins and erythrocyte membrane proteins. Banerjee et al (1974) reported that amino acid composition of cow and buffalo MFGM proteins were similar. Both membrane proteins were rich in aspartic acid and glutamic acid. In a comparative study, on membranes of rough endoplasmic reticulum, Golgi vesicles and milk fat globule, Keenan and Biaag (1973a) could observe some similarity in amino acid composition of proteins of these membranes. The proteins of the above membranes were rich in glutamic acid and leucine.

The studies conducted on the chemical composition of MFGM proteins show that glycoproteins are components of MFGM as in other biomembranes. The glycoproteins of MFGM are easily soluble after extraction of lipids from the membrane (Thompson and Brunner, 1980). Although it has been reported that the low density lipoproteins contained higher amount of carbohydrate than the high density lipoproteins (Swope and Brunner, 1970), Chien and Richardson (1967b) observed that hexosamines were distributed equally throughout the various lipoproteins separated based on density. Although presence of various inorganic ions in MFGM has been reported, no study has been published on the role of these ions on MFGM integrity or on protein-protein interactions in this membrane.
Electrophoretic pattern of MFPM proteins

Various workers (Brunner et al., 1963a; Brunner and Herald, 1968; Brunner and Thompson, 1961a; Jackson et al., 1953; Ramachandran and Whitney, 1960; Sasaki and Koyama, 1960a, b; Harwalkar and Brunner, 1968) have tried to separate the MFPM proteins by free boundary electrophoresis; but with little success. Their studies revealed the heterogeneity of MFPM proteins.

Keesan et al. (1970) in a comparative study of plasma membrane of lactating cell and MFPM noted that these two membranes had proteins of similar electrophoretic profiles with one major protein component in acidic gels. Analysis of membrane proteins of rough endoplasmic reticulum, Golgi vesicles and milk fat globules by sodium lauryl sulphate-polyacrylamide electrophoresis showed there were eight protein components which were common to all membranes (Keesan and Huang, 1972a). Several of these common protein components were major components in all above membranes. A protein component having molecular weight 68,000 was most prominent in all the membranes.

Anderson et al. (1972a) electrophoretically analysed the MFPM proteins solubilised in SDS-EDTA system and calculated the molecular weights of major separated components. The molecular weights of components varied from 16,000 to 2,20,000. They observed that ageing of milk resulted in the partial loss of the fastest
moving protein component (molecular weight 10,000), from deoxycholate soluble fraction of MFOM. They also stained for carbohydrate by periodic acid-Schiff reagent and detected eight glycoprotein components on MFOM. A number of components which were stained for carbohydrate were not stained for protein. Kobyla and Carraway (1972) separated the MFOM proteins by SDS-polyacrylamide electrophoresis and characterised the major membrane proteins and glycoproteins. Molecular weights of six major membrane proteins varied from 55,000 to 2,40,000 and they numbered the proteins in the order of decreasing electrophoretic mobility. Protein component VI appeared as a doublet in certain preparations. Seven components were stained for carbohydrate. The fastest moving component appeared to be a glycolipid while the others appeared to be glycoproteins. The major glycoprotein (glycoprotein 2) was invariable in terms of amount present in different preparations. It did not stain with Coomassie Blue. Apparent molecular weight of glycoprotein 2 changed from 2,00,000 on 5% gels to about 80,000 on 12% gels.

Electrophoretic analysis of MFOM proteins solubilised in SDS by gradient gel electrophoresis resulted in the resolution of MFOM into six major proteins and six major glycoprotein components (Anderson et al., 1974). The molecular weights of the proteins and those of glycoproteins varied from 1,60,000 to 18,000 and from
1,48,000 to 54,000, respectively. By separating the protein and glycoprotein components on several single strength SDS-polyacrylamide gels between 3 to 12% acrylamide concentration they calculated the free electrophoretic mobilities and retardation coefficients of proteins and glycoproteins. Free electrophoretic mobilities and retardation coefficients of membrane proteins III, IV and V and those of glycoproteins D, E and F were similar. Glycoproteins A, B and C had lower free electrophoretic mobilities. The authors pointed out that the protein component III and glycoprotein D, protein IV and glycoprotein E and protein V and glycoprotein F may represent the same species.

Banerjee et al (1974) observed that both cow and buffalo MFGM proteins resolved into 12 to 13 components, six of which constituted the major components, by polyacrylamide electrophoresis in acidic gels. The only difference in the electrophrogram of the above specimens was that buffalo specimen had a fast moving component which was absent from cow specimen. Brunner et al (1975) observed similarity in the electrophoretic patterns of delipidated specimens of cow and buffalo MFGM by SDS-polyacrylamide electrophoresis. Most of the protein species in the membranes were assigned molecular weights ranging from 54,000 to 1,00,000.

By employing two gel concentrations (8% and 10%), Mangino and Brunner (1975) have demonstrated the presence...
of approximately 17 protein components in MFGM fractions. The molecular weights of these zones varied from 13,500 to 2,80,000. The authors observed marked difference in the molecular weight profile of proteins in the original membrane and of those in the fractions obtained after extracting with 0.6 M KCl. The original material contained dominant protein species with molecular weights below 60,000, only traces of larger species were apparent. After extraction with 0.6 M KCl, there was a decrease in the lower molecular weight species and a concomitant increase in larger species. The protein components with molecular weights 48,000 and 1,00,000 maintained in their relative position in the gels although decreasing in concentration with successive KCl extraction.

Thus it is evident that there is overall agreement between the various published data on electrophoretic profile of MFGM proteins and glycoproteins. It appears that the membrane proteins I, III, IV and V reported by Anderson et al. (1974) are similar on a molecular weight basis to some of membrane proteins described by Kobylka and Carraway (1972). Molecular weights for principal zones of bovine MFGM reported by Mangine and Brunner (1975) agree well with those of Anderson et al. (1974). But Mangine and Brunner (1975) could not observe a major protein band equivalent to protein component I (Kobylka and Carraway, 1972) having a molecular weight of
Anderson et al (1974) similarly could not observe protein component VI (Kobylka and Carraway, 1972) having a molecular weight of 53,000. The above observations may be due to the difference in the procedure of isolation of MPGM or due to the difference in the source of the material used.

**Solubilisation studies on MPGM proteins**

The membrane proteins are components of highly interacted lipid-protein complexes and are quite insoluble in aqueous systems, thus difficult to isolate and characterise. Herald and Brunner (1987) reported that almost half the amount of delipidated MPGM proteins could be solubilised by 0.02M NaCl. The insoluble fraction was soluble in 25% sodium hydroxide, sodium sulphide and sodium lauryl sulphate solutions. Where solubility was achieved, an alkaline medium was required to retain the solubilised proteins in solution. Lyophilised insoluble MPGM proteins were not readily or completely solubilised by these agents. Alexander and Lusena (1961) reported that the MPGM material obtained by freezing the washed cream could be fractionated from suspensions in 2% sodium deoxycholate into five sedimentable fractions differing in chemical composition.

Relative solubilities of lipid-protein particulates, soluble and insoluble protein fractions in presence of various dissociating agents were studied by Harwalkar...
and Brunner (1965). The insoluble protein fraction was solubilised in solutions of guanidine hydrochloride + mercaptoethanol, 2% sodium sulphide and 2% sodium lauryl sulphate solutions. Sodium lauryl sulphate solution was less effective for solubilisation of insoluble protein fraction than the other two dissociating agents. Hence, the authors suggested that disulphide bonding is an important factor in the structure of insoluble protein fractions. Urea (7M) and guanidine hydrochloride (5M) could not solubilise the insoluble protein fraction.

The dissociation of intact, lipid-protein complexes by the above dissociating agents were similar to, but less extensive than the dissociation observed in delipidated proteins.

Hayashi and Smith (1965) reported that 48% of the total weight of the membrane surrounding the fat globules could be solubilised by sodium deoxycholate. The solubilised fraction was rich in alkaline phosphatase and xanthine oxidase (Hayashi et al., 1965). Depending upon their findings they suggested a structure for MFGE; the water soluble lipoproteins are adsorbed on a water insoluble matrix composed of lipids and proteins bordering the triglyceride core of fat globules.

Anderson et al. (1972a) observed that ageing of milk resulted in the loss of a protein component having molecular weight of 10,000 from the deoxycholate soluble fraction of MFGE. SDS, sodium deoxycholate, Triton X-100
and Brij-57 at 1% were observed to solubilise 41%, 20%, 13% and 9% proteins, respectively from MFGM (Hamag et al., 1972). Sodium deoxycholate was most effective in releasing the membrane bound enzymes 5'-nucleotidase and Mg\(^{2+}\) ATPase. Electron microscopic studies indicated that both Mg\(^{2+}\) ATPase and 5'-nucleotidase could be selectively solubilised from MFGM by deoxycholate under conditions which tend to leave basic structure of MFGM.

Kobylka and Carraway (1972) made an attempt to selectively solubilise MFGM proteins by EDTA, guanidine hydrochloride and 1M NaCl solution. They found some discrimination in the extraction of proteins from MFGM, but not as pronounced with red cell membrane. Protein component I did not appear to extract into EDTA at all, components V and VI were less readily extracted than III and IV. The major glycoprotein (glycoprotein 2) partitioned between extract and pellet. Glycoprotein 2 was primarily present in the extract. All of the MFGM proteins were observed to be extracted by sodium chloride and guanidine hydrochloride, i.e. no selective solubilisation was achieved.

Anderson et al. (1974) studied the effectiveness of various reagents in solubilising MFGM proteins from washed cream containing 50% (W/W) lipid. SDS, NaCl, sodium deoxycholate, butanol, dimethyl sulphoxide and Triton X-100 could solubilise 84%, 54%, 80%, 39%, 24% and
23% of membrane proteins, respectively. They found that gel patterns obtained from materials extracted by various solvents were similar, thus indicating no selective solubilisation of MFQM proteins was achieved by these agents. Mangino and Brunner (1974) have also reported that proteins extracted from preparations of MFQM by a variety of solvent systems showed negligible specificity relative to species solubilised.

The above studies on MFQM have shown that MFQM proteins are difficult to be solubilised in aqueous systems and to be characterised in absence of a dissociating agent. Although solubilisation of MFQM proteins have been achieved in presence of ionic and non-ionic detergents, the mechanism of solubilisation of MFQM proteins by these agents has not been reported so far. It is rather strange to note that MFQM proteins, unlike other membrane proteins, are not selectively solubilised by various membrane solubilising agents.

Structure and origin of MFQM

The milk fat globules, most of which are approximately 2.5 μm in diameter, are surrounded by a membrane (MFQM). MFQM is a complex mixture of glycerides, cholesterol, phospholipids and proteins accounting more than 1% of lipid phase in milk. King (1988), using chemical information, postulated MFQM as a monolayer of polar lipids, principally phospholipids, oriented on the fat globule surface and joined to one or more layers of
extended protein molecules, through electrostatic bonds. Morton (1954) observed with the aid of electron microscope small particles adhering to both intact and collapsed fat globules. He suggested that the fat globule is coated with a continuous layer of protein into which phospholipids containing microsomes of cellular origins are adsorbed. Hayashi and Smith (1965) selectively solubilised 45% of MFGM material by treating with sodium deoxycholate and observed that the soluble fraction (Hayashi et al., 1965) was rich in alkaline phosphatase and xanthine oxidase activities. They postulated that MFGM consists of an innermost layer of insoluble lipid-proteins to which soluble lipid-proteins are adsorbed. Chien and Richardson (1967a, b) supported the structure of MFGM proposed by Hayashi and Smith (1965) since they could also separate MFGM lipoproteins into various fractions based on solubility in the absence of a detergent.

Anderson et al. (1972a) reported that ageing of fresh milk produced changes not only in deoxycholate soluble fraction of MFGM, but also in low density membrane fraction as revealed by electrophoresis. In another study, Anderson et al. (1972b) observed that lactoperoxidase catalysed radio-iodination (I131) of MFGM resulted in the iodination of proteins in all the fractions separated based on the solubility of MFGM proteins in presence of sodium deoxycholate. These results
showed that all the fractions are partially exposed at the membrane surface and do not support the idea of well defined inner and outer regions of MFGM based on the solubility of major lipoprotein fractions proposed by Hayashi and Smith. Kobylka and Carraway observed that proteolytic digestion of milk fat globules with trypsin or pronase resulted in the cleavage of all the major associated membrane proteins. The above finding indicated that MFGM does not represent a significant permeability barrier to proteolytic enzymes and suggested that the membrane does not exist in an intact form on the globule. So they have cautioned the use of MFGM as a model for an intact plasma membrane.

Bargmann and Knoop postulated, based on their electron microscopic studies on MFGM, that during milk secretion, the fat droplet is progressively enveloped by the plasma membrane of the lactating cell and finally is extruded into the alveolar lumen. The above hypothesis was further supported biochemically (Brunner, 1968) and biophysically (Patton and Fowkes, 1987).

Dowben et al. (1987) detected the activities of many enzymes, including ATPase, which are usually associated with plasma membrane. Keenan et al. (1970) in a comparative study of plasma membrane and MFGM observed a close similarity between these two membranes with respect to proteins and lipids composition. Patton and Trans(1971) estimated the activities of enzymes characteristic of
plasma membrane on the surface of MFGM. They suggested that MFGM is a very good system to study the plasma membrane of the cells of mammary gland. Wooding (1971b) by electron microscopic studies showed that Golgi vesicles are also involved in the formation of MFGM during secretion. Keenan et al. (1970) and Morre et al. (1971) supported the hypothesis that membrane of Golgi vesicles is utilized for the formation of secretory vesicles membrane which fuses with plasma membrane and serves to replenish that portion of plasma membrane given up to fat globules.

Crescents of cytoplasm were observed to be caught between lipid surface and surrounding membrane at the time of fat globule secretion (Helmisen and Ericsson, 1968; Kuresumi et al., 1968; Wooding et al., 1970). The cytoplasmic materials containing varying amounts and kinds of cell organelles may be a source of enzymes detected in association with MFGM. Wooding et al. (1970) estimated that 1 to 3% of secreted milk fat globules in their study had crescents associated with them.

Keenan and Hsiang (1972a) observed that the membrane of Golgi vesicles was intermediate between that of endoplasmic reticulum and of MFGM with respect to the contents of lipid, phosphorus, cholesterol, protein bound sialic acid, cerebrosides, phosphotidyl choline and sphingomyelin. Fatty acid composition and electrophoretic pattern of the above membranes were similar. They postulated
that the Golgi apparatus derives its membrane from endoplasmic reticulum and transforms the membrane into secretory vesicles which are identical to and fuse with apical cell plasma membrane.

Knoop (1908) suggested post secretion dissociation of cytoplasmic membrane in the milk serum and the formation of a new interfacial layer by physical chemical adsorption of materials from the milk serum on the fat globules. Hansen et al. (1971) by electron microscopic studies showed that rearrangement of the components of MFOM took place after secretion; probably to attain a more stable structure. Patton and Fowkes (1967) postulated that Vander-Waal's attraction forces exist between the fat droplet and plasma membrane secretion and it is reasonable that hydrophobic forces of this type would continue to act after secretion; which may lead to rearrangement of the membrane.

Another type of post-secretion change in MFOM is loss of membrane material to skim milk phase. Wooding (1971b) in a detailed study on the structure of MFOM in whole milk observed that unit membrane is gradually lost after secretion. Bauer (1972) based on freeze-etching studies on MFOM proposed a model for the membrane. Within the lactating cell, the fat globules are enveloped by a macromolecular layer of phospholipids closely associated with several monomolecular layers of high melting triglycerides. During secretion, the fat
globules are enveloped by plasma membrane of lactating cells or with membrane material from Golgi vesicles. After secretion, the unit membrane is gradually lost again leaving phospholipid in a simple limiting membrane. Since galactosyl transferase, a marker enzyme used for Golgi vesicles, activity was not detected in MFPM, Keenan and Huang (1972b) also supported the view that certain proteins are deleted or undergo structural rearrangement resulting in loss of enzymatic activity.

Enzymes associated with MFPM

Various enzymes have been shown to be associated with MFPM (Bailie and Morton, 1958; Whitney, 1958; Brunner, 1965; Dobson et al., 1967; Patton and Trans, 1971). Some of the above enzymatic activities have been detected in almost all cell plasma membranes. The presence of these enzymatic activities on MFPM has strengthened the hypothesis that MFPM is primarily derived from the plasma membrane of lactating cell.

Morton (1953) observed that 30 to 40% of alkaline phosphatase activity present in milk was concentrated in cream, where it was adsorbed on the fat globules. Studies of Morton and associates (Morton, 1953b; Morton, 1954; Bailie and Morton, 1958) indicated that most of the enzymes associated with MFPM are of microsomal origin and they exist as lipoprotein complexes. They
identified alkaline phosphatase, xanthine oxidase, DPNH-diaphorase and DPNH-cytochrome C reductase in MFGM.

Quittenmeu et al. (1944) observed that two phosphodiesterases which differed in pH optimum and heat stability were present in milk. Zittle and Dellamonica (1988) reported that their partially purified alkaline phosphatase from milk possessed phosphodiesterase activity. Acid phosphomonoesterase activity has also been reported in MFGM (Dowben et al., 1987).

Tarassuk and Freinkel (1985; 1987) suggested that there are two lipases present in milk. The spontaneously active lipase was irreversibly concentrated in MFGM by rapidly cooling milk to 5°C and was named as membrane lipase. The lipase of normal milk which sedimented along with casein was designated as plasma lipase. They found that rancidity might develop in milk due to adsorption of lipase onto the fat globules. But Dowben et al. (1987) could not detect lipase activity in their MFGM preparations.

The association of xanthine oxidase with MFGM is well established (Ball, 1939; Morton, 1954; Zittle et al., 1986; Dowben et al., 1987). Herald and Brunner (1987) noted that while alkaline phosphatase activity was largely associated with the soluble fraction of MFGM, the xanthine oxidase activity mainly remained in the insoluble fraction. Most of alkaline phosphatase and xanthine oxidase activities were found to be lost.
during washing of fat globules (Zittle et al., 1988b). The xanthine oxidase activity was observed to increase linearly with fat content and also when milk was cooled from 30°C to 0°C. Back and Reuter (1973) reported that the activity of the enzyme increased when low fat separated milk was subjected to turbulent flow, probably due to the release of enzyme from combination with MFGM. The properties of xanthine oxidase in bovine milk were dependent on the state of the enzyme, i.e. whether free or bound to MFGM (Brailly and Eienthal, 1974). Oxidase activity of membrane bound enzyme towards NADH was enhanced relative to that towards xanthine. The authors observed that this reflected a change in relative Km values and enabled the ratio of xanthine to NADH activities to be used as a parameter for relative amount of free and membrane bound xanthine oxidase in milk fractions. Their results showed that half of xanthine oxidase activity was associated with a particular fraction representative of MFGM.

McMeekin and Polis (1940) and Polis and Shmukler (1950) observed that aldolase activity in milk was concentrated in cream and separator slime. Dowben et al. (1967) reported that aldolase activity in MFGM preparation was largely concentrated in supernatant fraction obtained after centrifugation. Kitchen et al. (1970) observed that 24% of catalase activity in milk was concentrated in cream. They detected the highest
specific activity of this enzyme in the precipitate obtained by centrifuging buttermilk at 10,000 x g for 15 min.

Adenosine triphosphatase is present in the plasma membrane of various cells (Fonting and Caravaggio, 1962; Raal and al., 1971). Dowben et al. (1967) detected Mg\(^{++}\)-ATPase as well as (Na\(^{+}\)-K\(^{+}\)-Mg\(^{++}\)) activated ATPase in bovine MFPM. The specific activities reported for the above enzymes were 0.74 and 0.91, respectively. Patton and Trans (1971) reported that they could not detect Na\(^{+}\)-ATPase in bovine MFPM. Huang and Keenan (1972b) observed that the ATPase of bovine MFPM was not inhibited by ouabain and not stimulated by Na\(^{+}\). This was contrary to the finding of Dowben et al. (1967) who reported that ATPase of MFPM was partially inhibited by ouabain. Huang and Keenan (1972b) demonstrated the lack of Na\(^{+}\)-ATPase activity in MFPM. An alternative explanation which they pointed out was the enzyme might be present in MFPM, but in an inactive form. The pH optimum and energy of activation of MFPM ATPase were found to be 8.5 and 10,200 cals/mole, respectively. The enzyme was activated by Mg\(^{++}\) and had a Km value of \(5.68 \times 10^{-5}\) M for ATP. With respect to Km, pH optimum, temperature effects and Mg\(^{++}\) dependence, the ATPase of MFPM resembled the activities in several other tissues (Skou, 1965; Emmelet and Bos, 1966, 1966; Parkinson and Radde, 1971; Spereleakis and Lee, 1971; Tanaka et al., 1971), but differed ATPase of other tissues in lack of
sodium dependence and lack of inhibition by ouabain.

5'-nucleotidase activity, which is widely used as a plasma membrane marker enzyme, in MFGM is well established (Patton and Trams, 1971; Huang and Keenan, 1971, 1972a). 5'-nucleotidase was partially purified from bovine MFGM (Huang and Keenan, 1972a). The enzyme was resolved into two fractions of activity by Sepharose 4B gel permeation chromatography. The first fraction of activity eluted from the column was designated as fraction V and the second as fraction VI. The pH optima in Tris-HCl buffer for both fractions were 7.0 - 7.5. The appearance of a second pH optimum at 10 in Tris-HCl buffer in presence of Mg$^{++}$ was observed for both fractions. The double pH optima in presence of Mg$^{++}$ was observed for both fractions. The above phenomenon has been previously demonstrated with 5'-nucleotidase from other tissues (Song and Bodansky, 1966, 1967; Levin and Bodansky, 1966; Burger and Levenstein 1970). Activation energies calculated for fraction V and fraction VI were 10.400 cals/mole and 9.400 cals/mole, respectively. The corresponding K_m values for AMP were 0.04mM and 0.02mM, respectively. The above K_m values were considerably higher than other previously reported values for 5'-nucleotidase from other sources (Song and Bodansky, 1967; Widnell and Onkeless, 1968; Song and Bodansky, 1966; Bosmann and Pike, 1971). HgCl_2 at 1mM concentration inhibited the
activity of fraction V and fraction VI by 41.7% and 60.8%, respectively. Mn⁺⁺ at 1 mM concentration activated only fraction V, but at 5 mM concentration inhibited the activity. Mn⁺⁺ at 1 mM and 5 mM concentrations inhibited the activity of fraction VI.

ATP was found to be a competitive and non-competitive inhibitor for AMP hydrolysis by fraction V and by fraction VI, respectively. Relative activities towards various substrates were different for fraction V and for fraction VI. The relative order of hydrolysis of nucleotides was CMP > IMP > GMP > AMP > XMP > dCMP dAMP > TMP and GMP > CMP > IMP > AMP > XMP > dAMP dCMP > TMP, for fraction V and for fraction VI, respectively. All of the above studies indicated that fraction V and fraction VI represented different enzymes.