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The Nature of the Protein Materials which Adsorb to the Fat:Serum Interface of Homogenised Milk

A Thesis

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SUMMARY

The adsorption of protein material to the fat/serum interface of the fat droplets of homogenised milk has been shown to result in the major denaturation of those proteins of milk which are adsorbed. The materials present on the surfaces of fat droplets of homogenised milks and of emulsions containing casein and fat and also of whey powder and fat were subjected to analysis by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis following solubilisation with sodium dodecyl sulphate or non-ionic detergents. Such materials showed no similarity to native milk proteins or native fat globule membrane proteins when similarly treated. While the techniques demonstrated the similarity of the natural fat globule membrane proteins of three natural milks, comparability did not exist among materials isolated from the homogenisation-created "membrane" materials of three homogenised milks. Similarly the "membrane" materials of globules having different size ranges within any one homogenised milk were not comparable.

It was concluded that the mechanical energy of the homogenisation process combines with surface adsorption energies to cause massive and varied changes to the properties of the native milk proteins which have become adsorbed to the homogenisation-created fat interface. The particular form taken by materials that appear upon a homogenisation-created membrane is apparently controlled by chance due to conditions of the micro-environment surrounding the fat-serum interface. There was no evidence to indicate that any one native milk protein was preferentially adsorbed.

INTRODUCTION

The Homogenisation Process and its Effects upon Milk Properties

The fat globules of milk range in diameter from below 0.5 microns to above 15 microns with an average diameter of about 3.1 microns though the average figure varies with breed of cows, stage of lactation and other factors. Upon homogenisation the average diameter of the fat globules is reduced to about 0.54 - 1.4 microns depending upon the effectiveness of homogenisation (Walstra, Oortwijn and de Graaf, 1969). There is an increase in globule surface area which can be calculated from the above figures as a change from 850 cm^2/ml in normal milk to 4950 cm^2/ml in homogenised milk which is an increase of 5.7 times (Walstra, 1969c). Various authorities quote increases of 4 to 8 times (Jenness and Patton, 1959; Trout, 1950; Brunner, 1965).

The fat/plasma interface that is created in this way is stabilised in some manner which results in an increase in the amount of nitrogenous material associated with the fat. Presumably protein from both milk plasma and the original fat globule membrane moves to the interface and acts as an emulsifying agent to stabilise the fat dispersion. The ability of milk proteins to act in this manner has been demonstrated in model systems containing butter oil, water, and pure milk proteins (Jackson and Pallansch, 1961). A soluble protein desorbed from the natural globule was very effective in reducing interfacial tension in the model system and the whey proteins were all shown to have a similar, though less extensive, effect. Micellar casein, however, does not depress free energy at the fat/serum interface and casein components would need to be activated in some manner if they were to adsorb to the interface.

The effect upon the properties of milk which result from the adsorption of a new protein membrane have been widely reported and reviews of the technological literature concerning homogenisation have been published by Trout (1950), Jenness and Patton (1959), and Brunner (1965). It is sufficient for the purposes of the present review to select a number of papers that illustrate the more important properties of the homogenisation-created membrane. These properties are:

A) Permeability to Lipase

Homogenisation of raw milk results in lipase activity whereas the normal membrane is usually impervious to this enzyme. As a bovine milk lipase has been detected as a minor component of casein (Fox and Tarassuk, 1968) lipase could become a component of a membrane which consisted of casein materials. The rate of the reaction of a lipase in an emulsion of triglyceride has been shown to relate to the area of interface present (Benzonana and Desnuelle, 1965) and if lipase is adsorbed during, or after, homogenisation this could explain the increase in activity of milk lipase which occurs as homogenisation pressures are increased (Frankel and Tarassuk, 1956). Lipase adsorption in model triglyceride emulsions is reversible (Benzonana and Desnuelle, 1965). Lipase adsorption can occur under much less energetic conditions than in the violence of homogenisation as is illustrated by the fact that pumping of raw milk is often sufficient to induce rancidity.

B) Reduction in Protein Stability

The stability of milk proteins is reduced after homogenisation. This is illustrated by the following Properties:

i) Heat Stability - The homogenisation of butterfat in milk will reduce the period of heating the milk can withstand before coagulating. This problem of

heat instability is complex because many factors influence it. Homogenisation could be influential through two effects. First, protein stability could be reduced through alteration of the salt balance, through the adsorption of ions, particularly citrate and phosphate, or through adsorption of individual proteins onto the newly created fat:plasma interface, or through the combination of both effects. Secondly, the fine dispersion of fat might provide weak points or structures upon which casein coagulates (Rose, 1963). Presumably the interfacial effects are important because model systems reconstituted from butter oil and skim milk can be stabilised by the addition of interfacially active mixtures such as emulsifiers to the butterfat so that effects of the fat phase upon heat stability of HTST-sterilized evaporated milk disappear at emulsifier levels above 4 grams per 100 grams of fat (Leviton and Pallansch, 1961). Apparently, the emulsifying agents preferentially adsorbed onto the interface and thereby prevented protein instability from this source. Furthermore, neither fat nor homogenisation are essential to reduction of heat stability of concentrated milks for the addition of inert materials with large surface areas such as activated carbon and filter paper will achieve this effect (Whittier and Webb, 1950).

In essence it appears that while the homogenisation process will reduce the heat stability of milks this need not be construed as evidence that the violence of the process is an essential factor but rather that interfacial effects resulting from the increase in surface area are dominant.

ii) Stability to Rennet Action - Upon treatment with rennet, homogenised milks coagulate more rapidly to produce a curd with lower curd tension and greater water binding capacity than normal (Trout, 1950; Peters, 1964). These observations seemed to indicate that a change in protein properties because of adsorption had occurred. If cream is separately homogenised and then added back to untreated skim milk, these effects can be reduced. (Tittsler and Ernstrom, 1965). Furthermore the reduction of curd tension can be achieved by homogenisation of skim milk at pressures above 2800 p.s.i. and this indicates that the violence of the process is sufficient to affect the proteins quite separately from the adsorption phenomenon (Iwaida and Tsugo, 1962). It thus appears that both adsorption and direct effects could influence the phenomenon.

iii) Stability to Alcohol - Casein is normally stable in the presence of up to 35% alcohol but this stability is somewhat reduced after homogenisation (Trout, 1950).

iv) Increase in Foaming Capacity - The tendency of milk to foam is enhanced after homogenisation. This effect can also be produced by homogenisation of non-fat milk which may mean either that surface active materials or the milk proteins have been altered in some way to enhance foaming (Tamsma, Konston and Pallansch, 1969).

v) Alteration of Nitrogen Distribution - The normal conditions of homogenisation do not influence the distribution of nitrogen in the various milk protein fractions (Trout, 1950) but severe homogenisation of skim milk and of caseinate solutions (2100 - 5700 p.s.i.

for 5 - 10 minutes) will cause a decrease in the casein nitrogen and an increase in the proteose-peptone nitrogen (Iwaida and Tsugo, 1962). Alteration in the form of casein micelles after severe homogenisation (3200 p.s.i. for 6 minutes) has also been observed by electron microscopy (Hostettler and Imhof, 1953). This change in form could indicate micelle agglomeration or the formation of fat/protein complexes.

In summary it appears that homogenisation does influence the proteins in some manner. It is, however, difficult to isolate whether the cause of these effects is the improved dispersion of fat, protein adsorption at an interface, direct protein modification, or some combination of these causes.

C) Susceptibility to Sunlight-induced Off-flavour - Sunlight-activated off-flavour is probably created by protein degradation and it is promoted by homogenisation (Brunner, 1965). Homogenisation pressures above 2000 p.s.i. are effective as are other processes such as pasteurisation, in promoting this defect. It may arise from degradation of a low density lipo-protein fraction of the natural fat globule membrane (Finley, 1968). Presumably therefore the influence of homogenisation might be simply the greater exposure of this component to sunlight because of the greater surface area of the homogenised fat globules. The retention of this natural fat globule membrane component in some form within the new membrane therefore seems to be indicated.

D) Susceptibility to Oxidation - Following homogenisation milk fat is less susceptible to fat oxidation which is normally copper catalysed. To explain this, three possibilities exist. It may occur because the surface concentration of copper is reduced as a result of the resurfacing for the

adsorbed protein binds less of this ion than the original surface protein does (Tarrasuk and Koops, 1960). It may be that reactive sites on the fat (especially the phospholipids) are protected from oxygen or catalysts (Brunner, 1965). Also there is a loss of phospholipid from the membrane (see below G) which may reduce susceptibility to oxidation.

E) Susceptibility to Freezing Destabilisation - Freezing is disruptive of many biological membranes including the natural fat globule membrane but the fat emulsion of homogenisation milk is relatively stable to this form of physical damage (Webb, 1968). If freezing damage is primarily physical due to the rigidity of ice crystals this might indicate that the homogenisation-created membrane is more robust but if freezing damage is primarily due to an increase in salt concentration of the serum as eutectic freezing progresses then it may indicate that the materials of homogenisation-created membrane are merely insensitive to procedures that would normally denature them.

F) Loss of ability to Cluster - Normal fat globules cluster under the influence of fat globules agglutinating factors thus promoting the creaming process. The material which promotes clustering contains at least two components. One of these is probably euglobulin which will adsorb onto normal fat globules but not onto homogenised fat globules. As homogenisation of a euglobulin solution does not destroy its ability to cluster fat globules in milk in which the natural agglutinin has been destroyed, this suggests that the membrane itself has altered and prevents the adsorption of the euglobulin. A second component of fat globule agglutinin is possibly phospholipid or lipo-protein and this is inactivated by homogenisation or vigorous agitation (Kenyon, Jenness and Andersun, 1966; Samuelson, Bergtsson,

Nilsson and Mattson, 1954). Homogenisation therefore appears to prevent clustering through a combination of the following: destruction of a component of the agglutinating material, surface alteration that prevents euglobulin adsorption, and physical changes such as increased viscosity that oppose globule association.

G) Retention of Phosphatides - A number of processing techniques result in a loss of phosphatides from the natural fat globule membrane (Koops and Tarassuk, 1959) and a mechanism by which loosely adsorbed lipo-protein units which contain a high proportion of phospholipid and are situated at the outer layer of the globule can be released has been proposed (Hayashi and Smith, 1965; Peereboom, 1969b). Homogenisation at pressures of up to 3000 p.s.i. promote this loss of phosphatides (Koops and Tarassuk, 1959) but at higher pressures the transfer of phosphatides to the serum is reversed, apparently due to incorporation of the phosphatides into a protein/fat complex having a density greater than 1 gram/ml (Greenbank and Pallansch, 1961). The sedimentation of phospholipid does not necessarily infer that formation of a fat/protein complex has occurred for lipo-protein units released from the natural membrane are themselves more dense than 1 gm/ml (Hayashi and Smith, 1965). These observations indicate that the homogenisation-created membrane of fat globules contains substantially less phosphatide per unit of fat surface than the original membrane (Tarassuk and Koops, 1960) and infer that the adsorbed lipo-protein units that cover the surface of the natural globule are replaced, or at least become less prominant, on the resurfaced globule.

H) Limitation of Re-surfacing capacity of the Milk - As fat content of creams is raised the effect of homogenisation is to decrease the stability of the fat emulsion so that

oiling off occurs. This effect becomes noticeable in creams containing above 20% fat and increases until at 80% fat the emulsion is broken by homogenisation (King, 1955; Webb, 1968). Presumably there is inadequate emulsifying agent to maintain the emulsion and probably some control of protein to fat ratio would be of value in stabilising creams for homogenisation.

Those fat globules of cream that are re-surfaced after homogenisation do preserve their individuality though they may floculate. However, as fat content is increased the efficiency of reduction in globule size is reduced (Goulden and Phipps, 1964). At pH values below 7 the material adsorbed differs from normal for the cream becomes more viscous as the globules attach to one another through the adsorbed materials (Dolby, 1957b).

Conceivably an insufficiency of available protein could lead to a re-surfacing that is inefficient (hence larger globule size) and is not entire, leaving hydrophobic areas on the surface and leading to eventual destabilisation.

I) <u>Variability of Viscosity</u> - Milk viscosity is observed to increase linearly as homogenisation pressure increases and this can be ascribed to purely physical factors relating to fat dispersion but may also be contributed to by some change in the nature of protein materials. Homogenisation of whole milk concentrates exaggerate the increase in viscosity but homogenisation of skim milk concentrates results in a decrease in viscosity (Sanderson, W.B., Pers. Comm.). This infers an alteration of the natural protein system which is distinct from adsorption phenomena and from the physical effect of the increased number of fat globules.

J) <u>The Stability of a number of other Properties</u> - A number milk properties do not appear to be influenced by the

homogenisation process. These include titratable acidity, pH, specific gravity, electrokinetic potential of the globules and freezing point (Trout 1950; Brunner, 1965). A common factor in determination of these properties is the salt balance of the milk which presumably therefore has not been measurably altered nor is there a release of acidic compounds. Nevertheless this does not preclude incorporation of sufficient ions to influence protein stability.

In summary it is possible to infer from the effects of homogenisation upon milk properties that the fat globules are re-surfaced by protein material and this process creates the following effects:-

i) Undenatured lipase is either incorporated within the homogenisation-created membrane or is able to adsorb onto it and penetrate it whereas neither of these actions was possible with the natural membrane.

ii) The proteins of milk are altered in some manner which is detectable as reductions of heat stability, curd tension, alcohol stability, and viscosity of skim milk concentrates, and by increase in foam production. The extent to which protein modification occurs is obscured by the contribution of the increased fineness of dispersion of the fat but the observations on skim milk show that protein stability is disturbed even in the absence of adsorption at the fat/plasma interface. Direct protein denaturation is possible as is exemplified by the inactivation of a component of the fat globule agglutinating material.

iii) The susceptibility of homogenised milk to sunlight activated off-flavour infers retention of the low

density lipo-protein upon the new membrane, though not necessarily in its original form.

iv) The antioxidant function of homogenisation probably infers that the fat is less available to either oxygen or catalysts of oxidation.

v) The created membrane resists rupture as assessed by freezing stability either because it is robust or is insensitive to further denaturation.

vi) The observed drop in concentration of phosphatides at the homogenisation-created surface indicates that the new membrane materials must themselves be efficient emulsifying agents.

vii) The capacity of milk serum to provide emulsifying materials is limited.

viii) The stability of properties related to the salt balance of milk infers that it is not greatly altered subsequent to membrane creation.

Direct Observations of the Nature of the Membrane of Homogenised Milk Fat Globules

Direct characterisation of all biological membrane materials has been limited by difficulties of dissociation and solubilisation. In the present study it is assumed that the important material of the homogenisation-created membrane is protein in nature and the study is restricted to these protein materials.

The Nature of Proteins that are likely to Provide Source Material for the Homogenisation-created Membrane

a) The Native Fat Globule Membrane - The nature of the milk fat globule membrane has been reviewed by King (1955), Jenness and Patton (1959), Brunner (1965), Prentice (1969), and Peereboom (1969b). The last two named reviews are particularly valuable for an understanding of the natural membrane in relation to modern views of the nature of biological membranes. An annotated bibliography has also been prepared (Anon. 1969).

b) Milk Proteins - Information about the proteins of milk has been reviewed by a number of authors in the book "Fundamentals of Dairy Chemistry" edited by Webb, B.H. and Johnson, A.H. (1965) and more recently by McKenzie (1970).

The Nature of Homogenisation-created Membrane Materials

There has been little study of this material. In a series of papers published in 1953 Brunner and co-workers described experiments that were extended in 1960.

In the first paper (Brunner, Duncan and Trout, 1953) creams were separated from homogenised and non-homogenised milks (apparently by normal separation procedures) and washed free of serum protein by serial dilution with distilled water at 50° and reseparated until the nitrogen:fat ratio in the

resultant creams fell to a steady level. With homogenised milk this occurred after only one washing but six washings were used because this number was necessary for the non-homogenised milk. The final nitrogen:fat value was higher for the washed cream of homogenised milk than for un-homogenised milk which was explained in terms of the increased surface area of the fat globules of homogenised milk. Repeated solvent extraction with ethanol and ether under conditions up to 40° for 15 minutes provided a protein residue that was assayed for amino acid content by microbiological assay. The authors recognised that the preparative conditions were rigorous and may well have altered the nature of the membrane materials but neverthe-less felt that this was the most satisfactory technique available to them.

The amino acid data showed distinct differences between the two types of membrane material; particularly in the levels of cystine, glutamic acid, and tryptophan, so it was concluded that "one or more of the protein components of the milk plasma had been adsorbed onto the newly created fat surfaces of homogenised milk".

Subsequently these materials were studied by use of Tiselius electrophoresis apparatus (Brunner, Lillevik, Trout and Duncan, 1953). The patterns obtained however had very thick lines and peaks were not sharp. This effect has been ascribed to the presence of fat in complex with proteins (Tobias and Gerf, 1959). The authors were not prepared to use this electrophoretic information to identify the materials of either the natural or the homogenisation-created membranes though they were confident that the membrane "did not represent the normal milk protein complement as one finds in milk".

The same materials were also studied by ultracentrifugal sedimentation diagrams (Brunner, Duncan, Trout and McKenzie, 1953). The sedimentation characteristics of the homogenisation-created membranes were not readily comparable with those of any of the pure milk proteins though "provisional" identification was made of "casein, Kekwicks whey protein, an altered lactoglobulin, beta-lactoglobulin, or a complex of two or more of the constituent proteins". The assumption was made that the sedimentation properties of the isolated materials could be compared with those of the native materials.

The only indisputable fact to emerge from the series of studies was that the natural fat globule membrane and the homogenisation-created membrane were quite distinct in their characteristics. Presumably adsorption of protein materials from the serum has occurred to cause the change in nitrogen:fat ratio and the amino acid composition.

That adsorption of whey proteins onto a fat surface is possible was demonstrated by emulsification of acid casein, whey and butterfat, followed by subsequent washing of the emulsion with 0.95% salt solution and paper electrophoresis of the membrane materials (Sasaki and Koyama, 1956). The adsorbed protein material migrated at the same rate as beta-lactoglobulin and was therefore identified as such. Casein and other whey proteins were identifiable in the unwashed creams but were not retained after washing so there must be doubt that they were ever adsorbed properly under the emulsifying conditions employed.

In an extension of the earlier three papers free boundary electrophoresis was employed to identify the adsorbed materials of an homogenisation-created membrane (Jackson and Brunner, 1960). In this study homogenised milk was separated, apparently in the normal fashion, the cream was washed three times with tap water, churned, and the fat and serum separated with a laboratory separator. The proteins in the resultant

serum were then salted out with 2.2 M $(NH_4)_2 SO_4$ and lipid extracted with ethanol and ether. (The recovery of materials from homogenised milk in this fashion was presumably very inefficient because normal milk separation will recover only about 50% of the fat globules of homogenised milk and churning of homogenised milk is similarly only about 50% efficient (Trout, 1950) so that only a proportion, perhaps a quarter of the globules could have released membrane proteins for subsequent salting out. Recovery of membrane materials was greater than previously reported but still could not have been quantitative). Following fat extraction the materials were dried under vacuum and then the recovered proteins were extracted with 0.02 M NaCl and centrifuged. Five fractions were isolated as follows:-

1) An insoluble fraction which formed a reddish brown pellet and when solubilised by peracetic acid, gave a single electrophoretic peak. Because of its colour, mucoidal appearance and insolubility it was identified as a pseudo-keratin and assumed to have its source in the natural fat globule membrane. (The creation of insolubility due to surface adsorption of other source materials was not considered though presumably this fraction constituted a large proportion of the extracted materials).

2) The material that was soluble in dilute salt solution showed a complex electrophoretic pattern so was further fractionated by adjustment to pH 4.6.

3) The resultant insoluble material was identified as casein because of its precipitation at the isoelectric point of casein and because after solubilisation in veronal buffer the electrophoretic pattern was not too dissimilar to that of casein. The dissimilarities that did occur were suggested to be due to a heat-induced beta-lactoglobulin: alpha-casein complex though, in raw milk, this possibility

was considered remote. (It is equally possible to infer that the dissimilarities to casein electrophoretic patterns are as remarkable as the similarities). By preparing artificial homogenates of casein, soluble membrane protein, and fat it was possible to reproduce a casein complex that was not too dissimilar to that extractable from homogenised milk. An interaction of these components to create this fraction of the membrane was proposed.

4) The material that was soluble at pH 4.6 was heated to 90° for 30 minutes and centrifuged at 25000 G for 30 minutes. The resultant heat denatured material in the sediment showed a single shallow electrophoretic peak and was assumed to originate from the whey protein fraction. (The reasoning behind this claim is not made clear).

5) The heat stable material that was still soluble also showed a single electrophoretic peak and was classified as "the soluble membrane protein" because after heating this protein (separately prepared) provided an ultra-violet scan pattern similar to that of the fractionated material. (Both patterns were however fairly normal for many proteins).

Throughout this study electrophoretic lines were thick and separation was not very sharp. Undoubtedly, however, the heterogeneity of the homogenisation-created membrane was established.

Another form of fat:protein complex was demonstrated by observing fat which sedimented on ultra centrifugation (Fox, Holsinger, Caha and Pallansch, 1960). The protein present in this complex, which accounts for about 20% of the fat in milk homogenised at 4000 p.s.i., was deduced to be casein on the

basis of three criteria. While the evidence showed that casein was present in the complex it did not show that whey protein was not also present.

Complex formation in artificial homogenates of calcium caseinate and fat was shown to depend upon the conditions present at the homogenisation valve and it was deduced that the violence of the process must activate the micelle. Also, the natural fat globule membrane materials were not essential to complex formation, and the fat-protein linkage was explainable in terms of van der Waals type association. However, calcium was involved in the complex and it was possible to complex 100% of the fat in sedimentable form by homogenisation after addition of calcium.

The extent to which parallels can be drawn between this complex and the fat:protein complex of re-surfaced globules is not clear. A range of protein:fat ratios exist having specific gravities between 1.02 and 1.19 and presumably this range extends through a range of lower protein:fat ratios taking the form of globules. If so, there is no indication that completely different associations need to occur.

The extent to which fat/protein complexes occur is not completely clear because lipo-protein units with densities of greater than 1 gram/ml are desorbed from the natural fat globule membrane by agitation (Hayashi and Smith, 1965) and would sediment under the conditions of experiment. These would contribute to, but not dominate, the fat levels detected if they did not themselves become involved in the complex.

Association of fat and protein has been demonstrated when milk is concentrated (Mabbitt and Cheeseman, 1967). Fat globule membrane materials extracted from concentrated milk and examined by polyacrylamide gel electrophoresis show two

components which are additional to the one that was found in the natural membrane. These were presumed to be $alpha_s$ and beta-caseins because they had similar electrophoretic mobility in polyacrylamide gel to that of these materials in native state. Further evidence that processing will create fat-protein complexes of varying densities and varying protein:fat ratio has been obtained with ice cream but this is not only an effect of homogenisation so a parallel cannot be too closely drawn (Wren and Bullimore, 1967).

Observation of the fat globules of homogenised milk by electron microscopy has indicated that the casein micelles adsorb to the fat interface so that some become spread around the globule and some retain in part a distorted micellar shape (Henstra and Schmidt, 1970). This information is not quite satisfactory for no comparable electron micrograph of the natural milk is shown. The fate of the whey protein cannot be deduced.

In summary it appears that little is proven about the nature of the material on the homogenisation-created surface because a number of difficulties have impeded the study. These are:-

First, the acceptance of the loss of the majority of fat globules by normal separation procedures and subsequent churning operations.

Secondly, no particular care has been taken to solubilise the membrane materials that are largely insoluble.

Thirdly, the assumption has been made in practically every case that materials adsorbed to the membrane and complexed with fat will still have the same electrophoretic characteristics as the native materials. If the protein is indeed as spread as the electron micrographs indicate

this assumption needs to be tested.

Fourthly, it is improbable that the density characteristics of membranes, still associated with some fat, will be comparable with those of native proteins so this approach is unlikely to be useful.

Finally, the techniques of protein chemistry in use by previous workers are much less sensitive than those available today and a re-examination of this information is now desirable.

The Process of Interfacial Denaturation and its Applicability to the Fat/Serum Interface of Homogenised Milk

The changes of properties to be expected after adsorption of milk proteins to the fat globule interface will presumably parallel those of other proteins that have suffered surface denaturation. Soluble proteins placed on a water surface or a liquid/liquid interface will spread to form insoluble mono molecular films in a process that is generally irreversible. Such denaturation is severe but the effects vary. Some enzymes are destroyed while others retain their biological activity and some proteins show no change in molecular weight while others dissociate into sub units (Joly, 1965).

Individual milk proteins too show different reactions at an interface. At a "butter oil/protein-free milk plasma" interface the soluble protein(s) of the milk fat globule membrane very effectively reduces interfacial tension. By comparison the globulins of milk are quite effective, the albumins of milk are slightly effective, while the large micelles of casein do not appreciably depress the free energy at the interface (Jackson and Pallansch, 1961). Conceivably the surface active proteins will concentrate at the homogenisation-created interface. If so, casein components will need to be released from the micellar form to become active. Possibly the violence of the homogenisation process can effect this activation.

Casein can be activated for it is able to contribute to the oil:water interface of ice cream. This product is homogenised but is different from milk because of the presence of stabilisers, an air/liquid interface, and agitation which leads to fat destabilisation so parallels must be drawn with care. Nevertheless it appears that in this product casein and gelatin can form a mixed interfacial layer in which casein is the dominant component in lowering the surface tension

(Musselwhite, 1966). Emulsifiers in ice cream adsorb to the fat/serum interface within seconds whereas proteins require tens of minutes or even hours, depending on their concentration. Adsorption from mixed solutions therefore yields surface layers containing several components according to the speed of adsorption and the ability of one component to replace another (Musselwhite and Walker, 1971).

The extent of unfolding of milk proteins at an air/water interface depends on a number of environmental facters. First, at low concentration the proteins unfold extensively, whereas increasing concentration leads to a variety of degrees of unfolding. Biological activity of enzymes is more likely to be retained in concentrated films where unfolding is less extensive. This occurs because unfolding occurs against surface pressure which is set largely by the numbers of molecules present. Secondly, the ability of a protein to spread is restricted by forces which will tend to maintain the native structure (e.g. the disulphide bonds of kappa casein reduce its surface activi y while alpha and beta casein spread more readily). Finally, denatured proteins (and presumably disordered proteins) probably have a greater number of hydrophobic residues exposed and so would anchor to an interface more readily than native proteins (Mitchell, Irons and Palmer, 1970).

In relation to the present study this information leads to the expectation that milk proteins will be massively denatured or even ruptured by adsorption to the homogenisation-created interface but the extent of alteration will vary with a number of environmental factors - particularly with the concentration. The prediction of which proteins will appear at the interface is probably idle and mixed layers of all available proteins (modified from the native form) is more likely.

Outline of the Investigation

The general aim of the investigation was to characterise the protein materials that occur on the surface of milk fat globules after homogenisation. To achieve this it was necessary to develop methods by which membrane materials could be recovered and then to characterise them by a number of techniques which would provide a range of information about them.

It was expected that the membrane materials of homogenised milks would contain proteins that originate from the natural fat globule membrane and/or the milk serum. It is possible that proteins of the milk serum might retain their natural properties after adsorption onto the surfaces of fat globules of homogenised milk and during subsequent preparative procedures in which case characterisation would be simple. It is, however, also possible that milk proteins may be so altered by the homogenisation process as to be unrecognisable on comparison with their original properties as natural proteins. To provide suitable "standard" materials of known origin for comparison with the membrane materials of homogenised milk, and also to investigate the effects of homogenisation upon the native milk proteins, studies have been made of natural fat globule membrane from whole milk and of "membranes" isolated from artificially prepared homogenates which contain only casein or whey protein as the protein source.

In outline the investigation has been performed in the following manner:

(1) Origin of Materials:

To ensure relevance to commercial processing operations milks were drawn from commercial sources.

(a) Whole Milks - Ten gallons of whole milk was drawn from bulk whole raw milk at the Palmerston North Milk Treatment Station on three occasions within one week. It is expected that chilling and agitation will have affected the nature of the fat globule membrane of these milks in

a manner that is probably common in commercial supplies of milk.

(b) Homogenised Milks - Within a month of the above, ten gallons of homogenised, unpasteurised milk was drawn from the same source as the whole milk on three occasions within a fortnight. Homogenisation had been performed as normally practiced by the Palmerston North Milk Treatment Station.

(c) Artificial Homogenates - Suspensions of casein and of whey were prepared and anhydrous butterfat was homogenised into them to form a stable emulsion.

(2) Preparative Procedures:

(a) Separation - The various milks were separated, to concentrate the fat into creams, by a farm milk separator. The three homogenised milks were each fractionated, on the basis of fat globule size, into two fractions. One fraction is comparable with normal milk and the artificial homogenate as it was separated in the normal manner (as above). The second fraction contained smaller fat globules that would not separate by conventional means, primarily because they had diameters less than 0.8 mM and these were separately prepared by use of more rigerous conditions of centrifugation on the "skimmed milk" from the milk separator. This latter fraction has been designated as the "minor" fraction in the text while the former fraction has been designated the "separable" fraction.

(b) Washing - The creams were washed by dilution with a solution of lactose in water followed by reseparation of the creams. This process was repeated until it was improbable that natural casein and whey proteins of the milk serum remained. The process was rigerous so that only strongly bound materials are likely to have remained.

Both of these requirements are necessary and consistent with the aims of the investigation. The washed fat globules were freeze-dried.

(c) Extraction of the Protein - Membrane Proteins were extracted from the freeze-dried cream powders by use of cold butanol and acetone by a technique often used for extraction of enzymes from cells. The commonly used process of churning the washed cream as a first stage in this process was not feasible for the small globules of homogenised milk will not churn properly.

(d) Solubilisation - So as to quantitatively release membrane materials, the prepared membrane materials were solubilised as effectively as possible in two different ways by use of anionic and nonionic detergents. The properties of both of the resultant detergent/protein complexes were then studied and results interpreted bearing in mind the limitations of the extent of solubilisation achieved.

(3) Characterisation on the Membrane Proteins:

(a) Chromatography of DEAE Cellulose - Column chromatography was used to separate the protein materials primarily according to their charge. This technique provides a semi-quantitative estimation of the amounts of material of different charge and also provides fractions that can be recovered for further investigation.

(b) Polyacrylamide gel electrophoresis - Electrophoresis in polyacrylamide gel was used to characterise the solubilised materials and also to indicate the nature of the various fractions separated by chromatography. It is more sensitive as a separation procedure because of its sensitivity to charge and its molecular sieving effect but large protein complexes are excluded from the small pores of the gel so that in this respect it is less satisfactory than the chromatographic technique.

The two techniques provide complementary information.

Techniques for Recovery of Fat Globules from Milks:-

Recovery of fat globules from milk is performed routinely by continuous centrifugation, the technology of which has been adequately described (McDowall, 1953). A proportion (less than 1.5% by weight) was, in practice, not recovered in this manner. The major reason for this was that globules having a diameter of less than 0.5 nanometer (nM) are too small for recovery by normal separating machinery (Kostygov and Rogov, 1966). Such small globules account for about 0.3% (by volume) of the fat in normal milk (Walstra, Oortwijn and de Graaf, 1969). Further fat losses arise from fat associated with casein micelles (Cerbulis, 1969) and from fat associated with a high density lipo-protein fraction (Finley, 1968) neither of which would be displaced into the cream fraction on separation.

Although this fat represented only a small loss, in terms of weight, the distribution of globule sizes was such that a large proportion (possibly 50%) of the total number of globules representing a significant proportion of the total surface area of globules could not be recovered (Walstra, 1969a). There was evidence that the very large numbers of small globules arose separately during secretion in the alveoli of the cow's mammary gland and may have had quite unusual properties (Walstra, 1969a, b). Such losses have always been accepted in studies of the milk fat globule membrane which therefore relate to the separable fraction. (See reviews by Brunner, 1965 and King, 1955).

While such losses may be acceptable with normal milk, the globules in well homogenised milk could have an average diameter of around 0.5 nanometers (Walstra, Oortwijn and de Graaf, 1969) leading to losses of 50% of the weight of the fat, and by inference an even greater proportion of the globules, when using normal separation procedures (Trout, 1950). The extent of such losses would vary with the extent of globule size

reduction achieved, which would itself be primarily dependent upon the pressure and temperature of homogenisation, and the condition of the homogenising valve, or alternatively the system of homogenisation (Goulden and Phipps, 1964; Walstra, 1966, 1969c). In addition to fat globule size reduction, homogenisation of normal milk at pressures near 2000 psi created fat:protein complexes, representing 10-15% of the total fat, that sedimented in the centrifugal field at a density of 1.026 gm/ml and so would contribute to fat losses (Fox, Holsinger, Caha and Pallansch, 1960; Cerbulis, 1969).

Losses of this magnitude have presumably been accepted in previous studies of the nature of the material absorbed on the newly formed fat surfaces consequent to homogenisation, for there is no mention of special precautions having been taken while separating the homogenised milks (Brunner, Duncan and Trout, 1953; Jackson and Brunner, 1960). In the present study an investigation of more effective separation techniques for homogenised milks was carried out for it was conceivable that the smallest globules, being more effectively subdivided, could have adsorbed protein materials differently to the larger globules. For example, larger globules might reasonably have carried a greater proportion of original fat globule membrane than the smaller ones.

For studies of the artificial preparations this consideration was of less importance for, no matter what size the globules were, they would present a clean fat interface upon which adsorption could occur. There would be no complication from material originating as native membrane. After preparation these materials were held in a milk can at 5° for 18 hours. During this period, incompletely homogenised fat and fat in large globules, would rise to the surface. After standing the top six inches of liquid was poured off thereby ensuring that only the adequately homogenised material was separated.

Techniques

1. **Origin and Separation of Non-homogenised Milks** -

Chilled bulk raw milk, obtained from the Palmerston North Milk Treatment Station was separated, at 37°, in an Alfa Laval, disc type, stainless steel, farm separator.

2. **Origin and Separation of Homogenised Milks** -

Chilled bulk raw milk was warmed to 45° and homogenised (two stages 1850/500 p.s.i.) and rechilled at Palmerston North Milk Treatment Station. Because fat hydrolysis by lipase was then possible it was transported immediately to the University and separation was begun within one hour.

There were two techniques -

(a) **Use of Sharples Super-centrifuge: (Type T 313)**

Various operating conditions were attempted. The most suitable conditions were: Bowl Speed, 40,000 r.p.m., Milk Temperature 45°, Inlet nozzle No.2 giving an inflow rate of 8.8 ml/second. Outlet dam: No. 7.5.

(b) **Use of Differential Separation** -

It was possible to fractionate globules into two size ranges by differential separation. The same principle has been applied to normal milks (Dolby, 1957).

(i) **Separable Fraction**:

The milk was separated by the Alfa Laval, disc type, farm separator. The separable globules were then washed and treated independently of the Minor fraction.

(ii) **Minor Fraction**:

Part of the "skimmed" milk, (about 600 mls) from the Alfa Laval separator was centrifuged in an MSE Major bucket type laboratory centrifuge at 5000 r.p.m. for 30 minutes in 100 ml bottles placed in an angle-head rotor. A cream separated out as a cake onto the inner wall of each bottle, so that the serum fractions

could be extracted by pipetting from the base of the bottle. Sediment also, was removed from the base of the bottle without disturbing the cake of cream. The entire operation was carried out at 37° in a thermostatically controlled room. This was consistent with the principles of efficient separation (McDowall, 1953) and also minimised oiling off of fat globules which could form a cake without being sufficiently warm to form a floating liquid layer.

Note:

Use of high speed centrifuges was considered but because yields of fat globule membrane material are small (about 0.04% of milk) it would have been necessary to handle larger quantities of milk than was possible in such equipment.

3. Preparation and Separation of the Artificial Preparations -

(a) Casein homogenate - Casein was prepared from skim milk by acid precipitation at pH 4.5. A Radiometer Automatic Titration apparatus using N. HCl was used and the fine precipitate obtained by centrifuging the slurry was washed twice with distilled water. The washed precipitate was re-dissolved in distilled water, adjusted to pH 8.0, and re-precipitated by addition of HCl to pH 4.6. This material was freeze-dried and stored at -15°C.

Five gallons of stable suspension containing 3% casein was prepared using distilled water, adjusted to pH 6.8, and with calcium added to give a concentration of 60 mgm % (Aiyar, 1969). After holding overnight at 5° to permit hydration, commercially prepared anhydrous butterfat was emulsified into the casein suspension at 50° to give a homogenate containing 8%

fat. This was done by passing the suspension twice through a single piston Empire laboratory homogeniser but after standing overnight at 5° some fat rose to the top of the liquid so the upper 10% of the liquid was discarded. Possibly casein alone is not a very satisfactory emulsifying agent, or possibly the homogeniser is unsatisfactory. After overnight storage at 5° homogenate was separated as for normal milk.

(b) Whey homogenate - Commercially manufactured lactic acid whey powder was used for this preparation. It contained 14% protein, 11.9% ash and 62% lactose and was reconstituted with water to give 8% solids in the reconstituted whey. It was considered to be casein free because insoluble casein fines were centrifuged out during its production. During manufacture the proteins may have been altered to some indefinable extent however as a consequence of the presence of proteinases originating from the mixed lactic cultures used or as a consequence of its being heated to 71° in the evaporators.

Whey powders prepared from rennet casein or cheese wheys were not chosen because their content of minerals would not be in the same balance as in milk (i.e. calcium would have been retained by the casein coagulum). A reconstituted and neutralised acid casein whey however provides all the normal milk constituents (except casein and fat) in nearly normal balance and so provides a suitable standard material.

Five gallons of reconstituted whey was prepared, NaOH added to raise the pH to 6.8, and held for 3½ hours after mixing to permit protein hydration and mineral

equilibration. Anhydrous butterfat of commercial origin was added to give 8% fat in the suspension. Homogenisation at 50° by two passes through a single stage homogeniser set to give nominally 2000 p.s.i. was performed. The homogeniser used was different to that which may have been unsatisfactory in preparing casein homogenate. The resultant fat emulsion was satisfactorily stable in that it gave the same fat test at top and bottom of a can after standing overnight.

After overnight storage at 5° the homogenate was separated as for normal milk.

Results of the Separation Procedures:

1. Separation of the non-homogenised milk was fully effective with loss of 1.2% of the fat.

2. Separation of the homogenised milk.

Table 1:

Treatment	Fat content of the milk fed to the machines (%)	Fat content of Effluent serum (%)
1. Sharples Supercentrifuge	3.3 - 4.1	2.0 minimum
2. Alfa Laval	3.3	1.2
3. N.S.E.	1.2	0.4 - 0.6
Cumulatively, 2 + 3	3.3	0.4 - 0.6

Note 1. Fat contents quoted were estimated by the Gerber Technique for homogenised milks (British Standard Method, 696 1955).

Note 2. The fat recovery achieved by use of the Sharples Supercentrifuge was poor and this was partly for mechanical reasons. The effectiveness of the machine rapidly decreased as sediment built up within the bowl and soon blocked off the serum outlets. These could only be cleared by heating in 1% phosphoric acid solution. Additionally the cream produced was badly destabilised

presumably because of the violent acceleration received by the globules upon entry to the bowl. For the purposes of this study the machine had no value so the differential system was employed.

3. Separation of the artificial systems -

Using only the Alfa Laval separator the fat level of the casein:fat homogenate was reduced from 8.2% to 0.3% while the whey:fat homogenate was reduced from 8.6% to 0.5%. These figures reflect the inefficiency of homogenisation achieved experimentally relative to that achieved commercially. There was no point in differential separation in these cases.

Discussion:

Because of the high speeds of the Sharples Supercentrifuge it had been anticipated that it would be more efficient than the disc type separator. The practical difficulties of operation noted above tend to obscure the true effectiveness of the Supercentrifuge but even so it apparently was no more efficient at separating the smaller fat globules. Having established appropriate operating conditions for the centrifuges it was possible to compare the separating efficiencies by employing the "Sigma Theory" approach (Trowbridge, 1962). According to this theory a value, Sigma, may be calculated which is equivalent to the area of a gravity settling tank of equivalent sedimentation characteristics to the centrifuge. This value is modified by the flow rate of liquid passing through the separator and the characteristics of the fat particles. It is then possible to calculate a value, D, which is the diameter of the "cut-point particle" (that is, D = the diameter of particles, half of which will be removed in passage through the centrifuge, and half of which will not be removed).

The various systems used in this study on milk separation are compared in Table 2. Details of the calculation of these values are to be found in Appendix 1.

Table 2. **Operating Characteristics of Separation Procedures**

Machine	Operation Conditions	Maximum Distance that fat has to migrate	Sigma Value (Σ)	Diameter of "Cut-Point" Particle (D)
Alfa-Laval	8000 r.p.m., throughput 100 ml/sec.	0.1 cm.	11.9×10^6	0.8 nM
Sharples	40,000 r.p.m. throughput 8.8 ml/sec.	1.1 cm.	8.5×10^6	0.3 nM
M.S.E.	5000 r.p.m. 30 minutes, batch system.	8.3 cm.	-	0.05 nM

Deduction from these figures would predict that the Sharples centrifuge should perform slightly better than the Alfa-Laval, for this limited purpose, but the improvement is of no value as it is negated by the practical difficulties previously outlines. The M.S.E. batch system did, in fact, give more effective separation as predicted by the above values but the serum after centrifuging still contained 0.5% fat. Therefore, about 16% of the fat is still not recovered. This does not indicate that a high proportion of this fat is in globules with diameters of less than 0.05 mM, for the calculated values at small particle sizes become inexact because of a number of limiting factors, namely:

(a) The drag effect of Brownian motion which slows the migration of particles (Trowbridge, 1962).

(b) As previously stated, homogenisation induced fat: protein complexes that had densities that exceeded 1.026 gm/ml. Presumably other complexes exist having densities ranging between 0.91 and 1.026 gm/ml.

(c) A proportion of fat in natural milk also is in a form that will not separate into a cream layer.

(d) The smallest resurfaced particles of homogenised milk may conceivably have rather different properties to the natural membrane as a consequence of the new surface. Such differences are not considered in the sedimentation formulae and could limit the application of the calculations.

Removal of Milk Serum Materials from the Separated Creams("Washing"):

The procedures used for purifying fat globule membrane material have been recently reviewed (Brunner, 1965; Swope and Brunner, 1968). In outline, following separation of a cream, contaminant materials originating from the serum are removed by repeated dilution and reseparation ("washing"). During this process, material which is loosely adherent to the fat globules can be lost to the dilution water or desorbed by agitation (Brunner, 1965; Prentice, 1969). It is therefore desirable to minimise the number of washes given consistent with adequate removal of contaminant materials. On the other hand adsorption of materials onto the membrane during handling is possible as exemplified by the absence of lipases in membrane of fresh milk (Dowben, Brunner and Philpott, 1967) but a lipase can be irreversibly adsorbed onto the membrane following cooling (Tarassuk and Frankel, 1957). The procedures adopted have necessarily been somewhat arbitrary and needed evaluation.

Electropherograms of the dilution waters show that serum proteins are practically undetectable in the fourth washing water (Swope and Brunner, 1968) and unadsorbed lipase is substantially removed after only two washes for it shows no activity when the washed cream is subsequently homogenised (Tarassuk and Frankel, 1957).

Because materials removed during washing are highly diluted, their detection is not reliable, so a more satisfactory means of evaluating the washing technique is to observe the material that remains after washing. This can be achieved by observation of the ratio of nitrogen to fat in the creams. This ratio falls as the contaminant serum proteins are washed away. A point can be selected at which this ratio is only slightly altered by further washing indicating that only the strongly adsorbed membrane material remains associated with the fat (Brunner, Duncan and Trout, 1953).

Lactose is included in the washing waters at a level

similar to that found in milk so that they simulate the composition of milk serum rather better than distilled water alone. The lactose also maintains the normal density difference between fat and serum thus assisting separation.

Additionally, it has since been shown that sucrose addition to washing waters reduces the erosion of some membrane components (Swope and Brunner, 1968) and also assists in recovery of enzymes in the active form (Dowben, Brunner and Philpott, 1967). It is expected that lactose would have a similar effect. Most previous workers in this field have extracted membrane materials by chilling, then churning the washed cream so the membrane was extensively ruptured so that 60 - 80% of the fat loses its globular nature (Brunner, 1965). Butter-oil (i.e. primarily triglyceride fats) was subsequently removed after melting the butter. The shattered membrane material was then concentrated, commonly by centrifugation, and some means of lipid extraction was subsequently employed (Brunner, 1965). Such an approach is of little value with homogenised milks since it is widely recognised that small fat globules will not readily churn (Trout, 1950). Churning acts essentially to rupture the membrane of the larger globules but this function can also be performed by freezing. Freezing of cream is known to destabilise fat globules so that approximately 30 - 40% of the fat will be destabilised on subsequent thawing (Webb, 1968). While the causes of this destabilisation appear to be numerous and their relationship to one another is not simple (Lovelock, 1957), studies by electron microscopy have shown that the ghosts of fat globule membranes appear as empty sacs without extensive fracturing of the membrane material (Dowben, Brunner and Philpott, 1967). Hence the method offers a technique which can be confidently expected to yield membrane material that has undergone a minimum modification.

Procedure:

A. Washing of creams that were separable by the continuous separator:

The separated cream was diluted (3:1) with a 4% solution of lactose in distilled water at 37°, stirred until dispersed and reseparated. This washing procedure was applied four or five times. The procedure took no more than three hours from the time of collection.

B. Washing of the minor fraction creams:

These creams were separated into cakes on the walls of the centrifuge bottles. They were diluted (approximately 5:1) with a 4% solution of lactose in distilled water, vigorously shaken then held for about 10 minutes to assist fat redistribution, and recentrifuged at 3000 r.p.m. for 30 minutes. This procedure was carried out five times in a room held at 37°.

The entire process of separation and washing was completed in a five-hour period and some lipase action had occurred.

C. De-emulsifying the washed creams:

The washed creams were poured into shallow trays and frozen within three hours in a deep-freeze cabinet at -15°C. The frozen material was transferred to a freeze-drier and dried at around 0° under a vacuum of 0.5 mm mercury for 2½ days.

D. Analytical evaluation of the washing procedures:

Nitrogen levels were determined by Kjeldahl procedure in general accordance with the New Zealand Standard Method for liquid milk and cream but modified to suit a micro-distillation procedure (for details see Appendix 2 .). To suit the procedure the cream powders

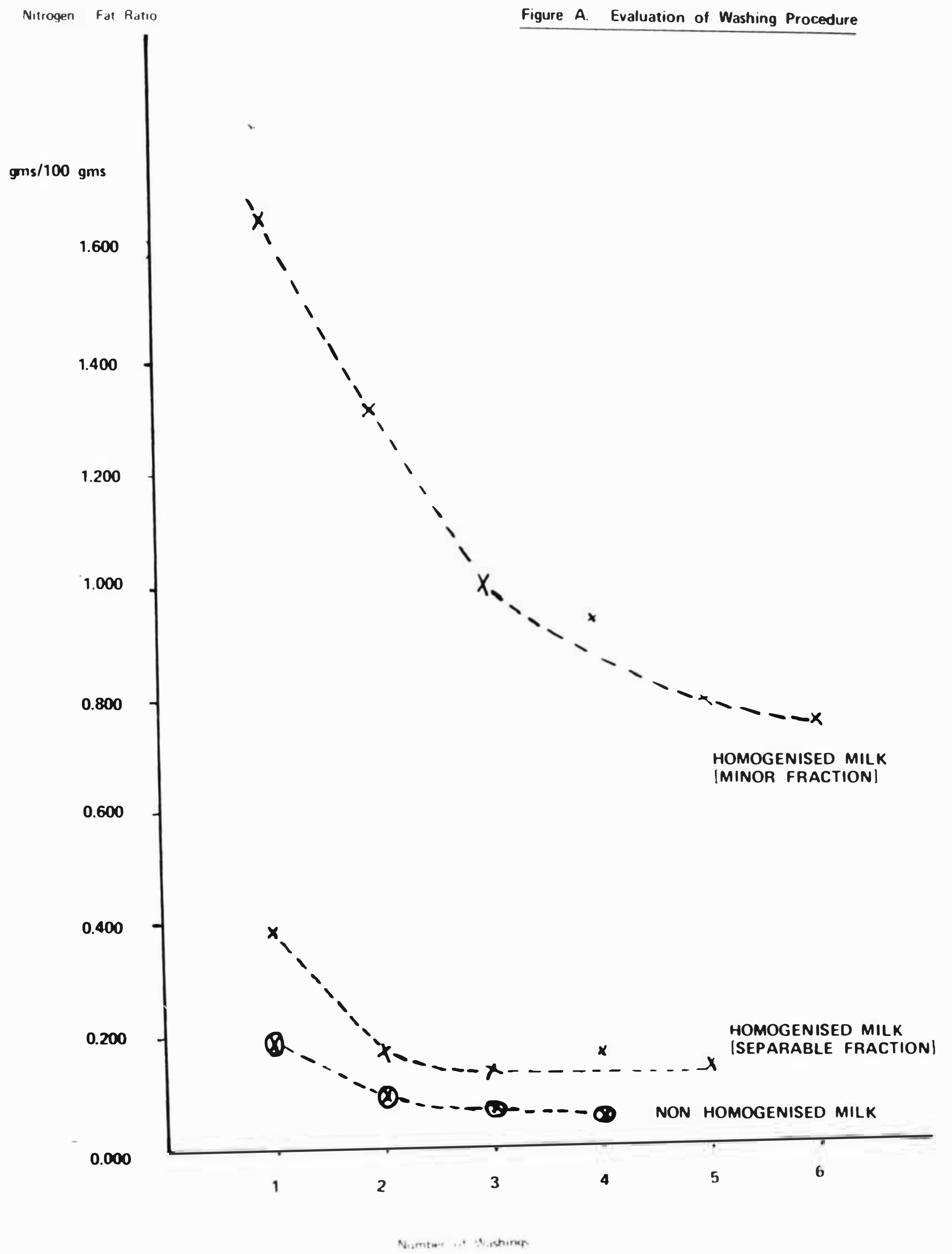


Figure A. Evaluation of Washing Procedure

were diluted to give approximately a 40% dispersion of fat in water (see too comment on use of Kjeldahl determinations in Section 9.).

Fat levels of the powders were determined by the Rose-Gottlieb method in close conformity with the New Zealand Standard Method NZS 2246:1969.

Results:

Figure A shows the effects of washing upon the various materials.

The nitrogen to fat ratio of the creams falls evenly as the washings continue until, after the fifth wash, a reasonably steady level has been reached and it may be accepted that plasma proteins have been removed.

Experimental materials were prepared in bulk on the basis of these findings and on analysis gave the following results (Table 3.) which also include the extent of variation found.

Discussion:

The nitrogen to fat ratios provide an indication of the efficiency of fat globule recovery in the various materials. The value for the separable fraction of the homogenised milk is a little greater (about 50%), and the value for the minor fraction is 9.5 times greater, than that for the natural membrane. This reflects the increase of surface area per unit of fat that occurs after subdivision of the globules. This is to say that on homogenisation the average diameter of the fat globules is reduced by about one-third which results in an increase of surface area (fat/serum interface) of approximately 5 - 6 fold (Jenness and Patton, 1959 and also calculable from the observations of Walstra et al, 1969 and Walstra, 1969b). The observed increase in the proportion of nitrogenous material associated with the fat provides evidence that protein adsorption

Table 3.

Sample Origin	Treatment	No. of Washes	No. of Preparations	Nitrogen/fat Ratio	
				Av.	Range
A. Non-homogenised milk	Alfa-Laval	5	4	0.073	0.045 - 0.099
Bi. Homogenised milk	Sharples	5	1	0.035	-
Bii. Homogenised milk	Alfa-laval	4	4	0.119	0.109 - 0.136
Biii. Skimmed fraction of the above (Bii)	M.S.E.	5	4	0.688	0.533 - 0.786

at the newly formed interface has occurred. The smaller globules of the minor fraction (Biii above) have adsorbed more protein per unit of fat and so provide a richer source of adsorbed material than does the separable fraction (Bii above).

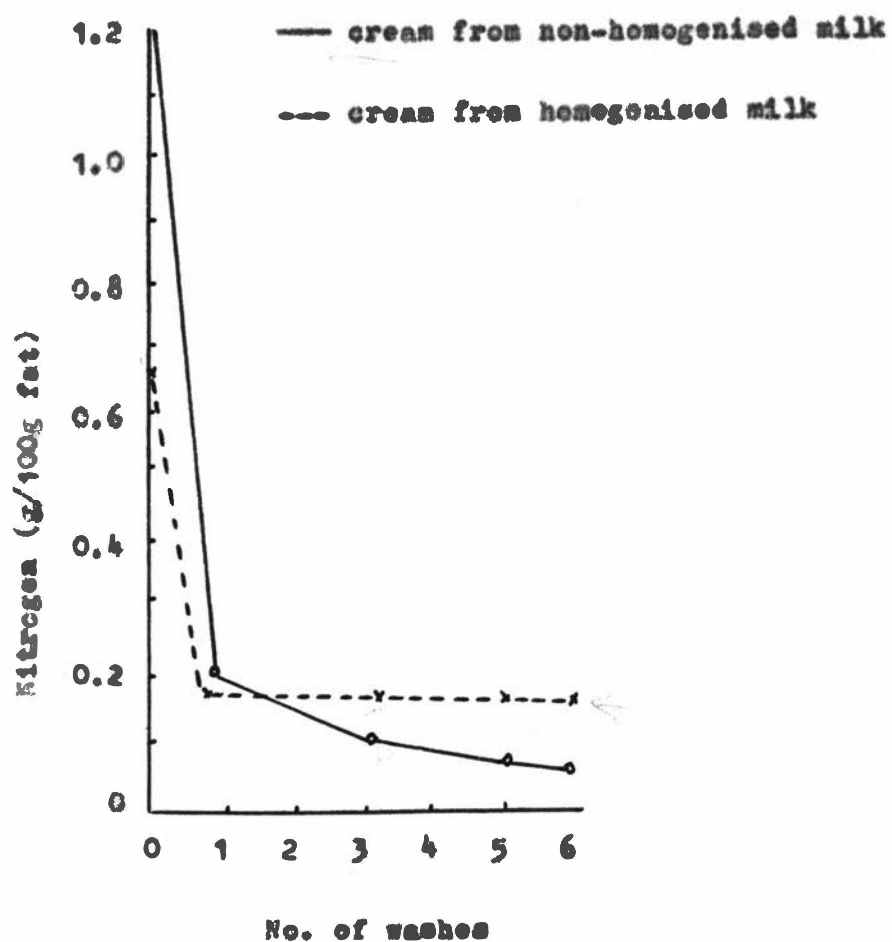
The observed nitrogen to fat ratios illustrate the ineffectiveness of the Sharples Supercentrifuge system because globule destabilisation occurred during washing and must have caused a considerable loss of membrane protein to the serum, but oiled off fat has concentrated into the creams thereby depressing the nitrogen to fat ratio.

The decline of the nitrogen/fat ratio for the cream minor fraction is less rapid than that of the other fractions and this probably reflects the means of handling (batch separation vs continuous) rather than any change in properties of the materials. The heights of the initial fat/nitrogen ratios in each case reflect merely the fat tests of the initial creams when separated.

The only comparable evaluation of the washing procedure for homogenised milk that has been published shows a different pattern (see Fig. B) although the pattern for normal milk is substantially the same (Brunner, Duncan and Trout, 1953).

The low final level of the nitrogen to fat ratio reported by these workers for homogenised milk reflects the less complete separation achieved and is reasonably comparable with the globules of the separable fraction in the present study. The ineffectiveness of the washing procedures after the first wash is unexplained. It could be inferred that the membrane material of the homogenised milks is tightly bound and does not erode on continued washing. This explanation is inadequate for continued washing steps should result in serial dilution of plasma proteins that must still remain with the cream after the first wash. Electrophoresis of first wash water from natural membrane preparations shows that this would be so (Swope and Brunner, 1968). The more gradual decline of the nitrogen/fat ratio found in the present study is considered

Figure B. Effect of Washing (Brunner, Duncan and Trout, 1953)



reasonable.

The number of washings that have been employed by various workers has been as low as one (e.g. Finley, 1968) and as high as twelve (Chien and Richardson, 1967) but is commonly three (Brunner, 1965). In the present study three washings would have been inadequate for the homogenised minor fraction so four and five were used (as shown in Table 3.). The choice of end point is somewhat arbitrary and in this study only those materials that are strongly adsorbed to the fat interface can remain. It is most unlikely that loosely held or occluded plasma proteins will remain to confuse the final conclusions. Similarly, loosely held, naturally occurring materials will have been removed so it is recognised that the extracted materials are neither quantitatively nor qualitatively identical with those originally present.

Section 7

TECHNIQUE OF EXTRACTION OF THE PROTEIN MOIETY FROM THE LIPID:PROTEIN COMPLEXES OF THE VARIOUS WASHED-CREAM POWDERS

Introduction

The various requirements for the extraction are first the fat in the powder has to be removed as completely as possible, secondly the various lipid:protein bonds occurring in the experimental materials have to be broken so that an aqueous suspension of the protein materials can be made, and thirdly, there must be only minimal damage to the protein material.

To meet these requirements is not simple and a number of considerations arise. These are:

(a) Techniques previously used with natural milk fat globule membrane material have possibly been too severe -

The techniques for isolation of milk globule membrane material have been recently reviewed (Brunner, 1965).

The difficulties of extraction of the protein moiety of the milk fat globule membrane are illustrated by the variety of exhaustive techniques that have been employed, though there is little information about the possible effects these treatments may have upon the nature of the protein moiety.

Various solvent systems have been tried. They are:

(i) Use of ethanol in ether

Lipid extraction of fat globule membrane material was carried out, in a classical study, by refluxing the material with hot absolute ethanol followed by hot extractions with absolute ethanol in anhydrous ether which was continued until fat was no longer detectable in the extracts (Palmer and Wiese, 1933).

The technique was later improved by using the solvents cold (5°) (Brunner, Duncan and Trout, 1953), and since then the same solvents have been very commonly employed (Herald and Brunner, 1957; Jackson and Brunner, 1960; Swope and Brunner, 1968; Finley, 1968).

(ii) Use of Chloroform, Methanol and Acetone

A recently introduced alternative has been extraction with chloroform:methanol mixtures including 2% KCl solution, followed by three washes with the solvent mixture at room temperature, and finally an acetone rinse (Swope and Brunner, 1970; Swope, Rhee and Brunner, 1968).

(iii) Use of n-butanol

The use of n-butanol as a solvent was investigated but rejected in favour of 35% ethanol in ethyl ether on the grounds that lipid levels of the material extracted by the n-butanol were higher and solubility in water was consequently lower (Herald and Brunner, 1957). Presumably the less effective fat removal caused the observed lower solubility in water. Both xanthine oxidase and alkaline phosphatase showed measurable activity in the extracted materials after either treatment. N-butanol alone has been successfully used to extract, and to investigate, the three forms of alkaline milk phosphatase (Peereboom, 1969).

(iv) Use of acetone, ether and butanol

The use of acetone and ether extractions, under anhydrous conditions, at 5°, was useful in removal of fat and a further treatment with n-butanol, at 5°, was effective in further reducing the lipid level of prepared membrane material (Zittle, Dellamonica, Custer and Rudd, 1956). However,

these conditions did lead to some loss of enzyme activity, particularly that of xanthine oxidase.

(b) <u>The adsorbed material on the fat/serum interface of homogenised milk and artificially prepared systems may be more sensitive than natural membrane to Solvent action</u>

A fat:protein complex, isolated from homogenised milk, and having a density greater than that of milk serum has been investigated with respect to stability to solvents (Fox, Holsinger, Caha and Pollansch, 1960). Treatment with n-butanol caused much of the material to sediment more rapidly in an ultracentrifuge than sedimented in an untreated sample which would be consistent with an assumption of removal of lipid from the complex with consequent increase in density. Treatment with 25% ethanol in ethyl ether, however, destabilised most of the faster sedimenting material of the complex and this formed a clear gel, which could not be redispersed in veronal buffer, in the bottom of the centrifuge cell. Avoidance of this latter solvent system in the present study is therefore indicated because materials adsorbed to the fat interface by homogenisation could show the same effect, even though this solvent system has been accepted for studies of the natural fat globule membrane.

(c) <u>Extensive studies of the membrane material of blood erythrocytes have shown that n-butanol is a suitable solvent for that material</u>

(i) <u>The use of n-butanol alone</u>

The use of n-butanol to isolate the protein moiety of natural membranes has been thoroughly investigated during work with the membrane material of blood erythrocytes. The technique used was that of Maddy (1964, 1966) in which

n-butanol at -3° was shaken with an aqueous suspension of insoluble erythrocyte ghosts. The butanol-saturated aqueous phase was centrifugally separated. A mean recovery of 83.1% (S.D. $\pm$ 3.8%) of the membrane protein in soluble form in the butanol saturated aqueous phase after only a single extraction was possible (Rega, Weed, Reed, Berg and Rothstein, 1967). Furthermore, the use of n-butanol resulted in a minimal increase (5%) in p-chloro-mercuric-phenylsulphonate-titratable sulphydryl groups and enzymes that normally reside in the membrane were recovered to the extent of between 75 - 107% after butanol treatment. Such reduction of enzyme activity as was apparent (60% loss in one case) was interpreted to indicate that the enzymes affected depend on the presence of an intact lipid-glycoprotein complex for their activity rather than that they suffer a specific denaturing effect due to the n-butanol.

The amino acid composition of the untreated erythrocyte ghost material was not significantly different from that of the butanol solubilised material (Zwaal and Van Deenen, 1968a). N-butanol solubilised proteins have been used to investigate the differences in the erythrocyte membranes of various animal species by observing the patterns produced by disc electrophoresis (Zwaal and Van Deenen, 1968b). Centrifugal fractionation followed by a second electrophoresis of the solubilised material indicated that artifact formation had not occurred. There can be little doubt that species differences observed in such a comparative study were real.

(ii) The desirability of combining n-butanol with a second solvent

Although butanol was an excellent fat solvent removal of fat from the erythrocyte ghosts was not complete, for 3% of the phospholipid remained along with the protein in the aqueous phase (Zwaal and Van Deenen, 1968b) and, in particular, phosphatidyl serine was poorly extracted. Acetone, and also methanol at 50°, subsequent to butanol treatment, have been used to improve lipid removal (Schneiderman and Junga, 1968).

The use of acetone at low temperatures (-15°C) is recommended for recovery of enzymes with maximum activity (Morton, 1955) and it has been employed to isolate protein from the membrane system of the chloroplast (Criddle and Park, 1964).

(iii) The unsuitability of many solvents for study of the erythrocyte membrane

In contrast to the above experience there has been unsuccessful use of ether, chloroform, ethanol, heptane and toluene to defat erythrocyte ghosts at room temperatures or below (Azen, Orr and Smithies, 1965).

(d) The Choice of a Solvent System

In view of the above considerations the chosen technique was based on extraction with n-butanol and acetone as employed commonly for extraction of enzymes from biological materials (Morton, 1955). Confidence in the technique had been established in a study of cheese proteins (Fenwick, 1953) and the results obtained were fully comparable with those of Melachouris and Tuckey (1966) and of Richardson and Creamer (1969) who used different extraction techniques, though each has interpreted their data differently.

Procedure

A small amount of freeze-dried, washed, cream-powder, (never more than 20 grams), at -15°, was mixed in a pre-cooled Waring Blendor jar, with about 150 ml of n-butanol at -15°, at medium speed for three minutes. The mixture was poured into pre-cooled centrifuge bottles and centrifuged at 2800 r.p.m. for 8 minutes in an International centrifuge (Model UV) and the resultant clear butanol layer decanted without delay and discarded. As soon as possible, about 150 ml of acetone at -15° was added to the centrifuge bottles and, with the protein precipitate, was transferred to another pre-cooled Waring Blendor jar in which it was mixed for a further 1 minute. Without delay, to ensure that the mixture remained cold, it was filtered through Whatman No. 50 paper on a buchner funnel. The resultant powder was held under vacuum in a desiccator until all trace of acetone was volatilised. The powder was then held (for not more than 2 months) in the dark, in stoppered bottles, at -15°.

Discussion

During this procedure, the treatment with n-butanol alone under non-aqueous conditions, gave a non filterable solvated material which still contained a proportion of fat as indicated by the appearance of yellow colour and fat that could be subsequently removed with acetone. Further treatment with acetone produced a dry powder that could be readily handled and stored and it also reduced the fat content of the material.

The resultant powder did not contain excessive lipid but it did contain some high melting point fats (M.P. above 40°). It has been widely observed that fat globule membrane material is often associated with a high melting glyceride fraction (e.g. Brunner, 1965). The origin of such material is open to question for, in prepared globules containing only tripalmitin and tricaprin, cooling caused crystallisation of the higher melting point molecules at the globule surface, (Bucheim, 1970),

and this also appears to occur with the high melting glyceride fraction of butterfat on cooling. The high melting glyceride fraction is probably not an integral part of the natural fat globule membrane (Vasic and De Man, 1966). In this study, high melting point material was found when lipid-protein membranes (both natural and artificial) were prepared after a cooling step, and its removal by solvents was difficult. The materials of the high melting glyceride fraction are probably insoluble in acetone (Mattsson, Swartling and Nilsson, 1969). Presumably this lack of solubility has given rise to the need for multiple extraction procedures as commonly employed. Removal of this material was readily achieved during subsequent solubilisation procedures reported later in this study.

<u>Summary</u>

The main points arising from this investigation are:-

(1) The freeze-dried cream powders have been fat-extracted by single sequential treatments with n-butanol and acetone at low temperatures.

(2) This technique produced membrane materials in powder form which still contained a small amount of high melting point glycerides which can be readily removed at a later stage of processing.

(3) Various workers have successfully used forms of this technique for enzyme extraction studies and for studies of the membrane proteins of erythrocytes.

(4) Previous studies of the milk fat globule membrane have employed repeated extractions with ethanol in ether but there is evidence that this system would not be satisfactory for materials from homogenised or artificial systems.

SOLUBILISATION OF THE MEMBRANE MATERIALS

The prepared membrane materials are difficult to solubilise for further analytical investigations. Natural membranes show insolubility under physiological conditions as a common property for they have a structural role to play in their native environments. The membrane proteins are in a class of their own in that they lack crosslinking amino acids, they have a high proportion of hydrophobic amino acids, and the amino acids present are predominantly acidic (Benson, 1968). Insolubility of the natural fat globule membrane is therefore to be expected for its origin may well be in the plasma membrane at the apex of the secretory cells of the mammary gland (Patton and Fowkes, 1967). A serious bar to investigation of membrane materials is that the analytical procedures commonly used in the study of proteins require that the material be in soluble form. Solubilisation had therefore to be effected as completely as possible.

Milk fat globule membrane materials have been most satisfactorily solubilised by use of either 5 M. guanidine hydrochloride plus 2-mercaptoethanol, or 2% Na_2S, or 2% sodium dodecyl sulphate (Harwalkar and Brunner, 1965). In no case, however, was complete solubilisation effected so an "insoluble fraction" had had to be accepted as an uncharacterisable fraction of the membrane. This fraction could be of pseudo-keratin nature (Brunner, 1965). The membrane material probably contains a number of lipo-protein units (Chien and Richardson, 1967) the solubilisation of which will presumably be influenced by the extent of lipid removal. The milk fat globule also carries water soluble materials such as a mucoprotein which contains sialic acid, hexose and hexosamine (Jackson, Coulson and Clark, 1962). Lipo proteins that are water soluble are also present (Hayashi and Smith, 1965). Being watersoluble these components are unlikely to cause difficulty and some solubility is to be expected with all dispersants. Because

of its likely origin and because of its general membrane properties, comparison of the fat globule membrane with other naturally occurring membranes is probably of relevance to this study.

The problem of partial membrane insolubility has been overcome in studies of erythrocyte stroma by use of 2-chloroethanol at low pH, whereby complete solubility was achieved and electrophoresis of the components solubilised was possible but there was loss of enzyme activity (Zahler, 1968). This technique has been used in the present study and it will successfully disperse the insoluble material of the natural fat globule membrane. The dispersed material however would not enter the polyacrylamide gels used for electrophoresis so its promise could not be realised.

The membrane material is therefore complex and a combination of solubilising techniques may be required. The materials to be solubilised from the fat/plasma interface of homogenised milk and of artificial preparations of fat and casein, and of fat and whey protein, are e en more complex and difficult to disperse. These various homogenates may well contain membrane materials arising from natural fat globule membrane or its fragments, natural or denatured casein and whey proteins, and lipid/protein complexes induced by the homogenisation processes.

The following table summarises the types of bonding that occur, or potentially may contribute to the complexes and gives the means of disrupting these bonds which were employed in this study.

Bond Type	Occurrence	Proposed Dispersant Procedure
Electrostatic bonds a) Ionic	Lipoproteins - Ion pair bonds in lipo proteins are feasible but not likely to contribute significantly (Cornwell and Horrocks, 1964).	

	<u>General Observations</u> - Fatty acid anions crowd to a phase interface and contribute to electrostatic fields (Gurd, 1960). Ionic linkages between phospholipid and protein have been shown to occur within the mitochondrial system where the complex is considered to be both electrostatic and non-polar. <u>Natural Fat Globule Membrane</u> - Polar lipids (especially phospholipids) concentrate at the globule surface. They are solvated and/or could be associated with charged groups of proteins (Brunner, 1965). <u>Artificial System</u> - Studies of artificial phospholipids: beta lactoglobulin systems have shown that interactions probably do occur (Payens, 1959).	pH (alkaline)	
b) Salt links	The natural fat globule membrane contains Magnesium which may contribute to association of the material through salt linkages (Peereboom, 1969). However, neither calcium nor magnesium is concentrated on the membrane relative to the serum (Swope & Brunner, 1968) so the extent of this form of	Ethylene diamine tetra acetic acid di-sodium salt, (Na_2 EDTA) at alkaline pH.	

	linkage may not be great. EDTA will dissociate a fraction of the protein material from the complex of the erythrocyte membrane (Rosenburg and Guidotti, 1969) and possibly a parallel can be drawn. Materials sedimenting from homogenised milk - Such materials, which include casein, and fat:protein complexes, are readily dissolved for subsequent fat extractions by soaking in EDTA (Fox et al, 1960). Milk Protein - Salt links within the casein micelle through calcium and phosphate, occur and contribute to micelle size and stability (Tumerman and Webb, 1965).		
c) adsorptive	All globules have a native charge which varies with pH suggesting ready adsorption of ions (Tjepkma and Richardson, 1967). It may be considered to have colloidal properties of adsorption (Payens, 1964).		
Hydrogen Bonds	Natural Fat Globule Membrane - 5 M guanidine hydrochloride and 7 M urea assist dispersion of membrane material and so indicate the importance of H bonds to this material (Harwalkar and Brunner, 1965). Natural lipoproteins - Natural lipo proteins are not likely to contain H bonds (Cornwell and Horrocks, 1964). Adsorbed Proteins - Proteins adsorbed at interfaces have probably unfolded which would cause rupture of intramolecular	Urea	

	H bonds and yield sites for intermolecular H bonds between protein and polar lipids. Water molecules would, however, compete for H bond sites (Cornwell and Horrocks, 1964). <u>Casein</u> - Urea is valuable in dissociating the casein complex prior to electrophoresis which indicates that the complex stability is dependent on hydrogen bonding (Wake and Baldwin, 1961).		
Hydrophobic Bonding	<u>Proteins</u> - Intramolecular hydrophobic bonds between non-polar amino acids contribute strongly to stabilising the folded configuration (Kauzmann, 1959). <u>Lipo proteins</u> - Intramolecular lipid:lipid and lipid:protein hydrophobic bonds are probably very important in lipo protein association (Cornwell and Horrocks, 1964). A non-ionic detergent, Triton X-100, (a polyoxyethylene ether of an alkyl phenol) combines with the lipid moiety of the alpha-lipo protein of blood serum and removes it from the association (Scanu and Oriente, 1961).	Detergents. (Urea may possibly weaken these bonds, (Kauzmann, 1959).)	

	<u>Natural Fat Globule Membrane</u> - Dissociation by sodium dodecyl sulphate is effective and also the non-ionic detergent Amidox 10L (an ethoxylated alkylo-amide) is of some value. This infers that hydrophobic bonds are important to membrane integrity (Harwalkar and Brunner, 1965).		
	<u>Associated Problem</u> - Acetone insoluble, high melting point (about 43°) lipids are associated with the membrane materials of this study and reduce effectiveness of dispersants possibly by preventing contact with the material. Lipids have been found to float free once melted (See Sections 7 and 8.2).	Warm to 50°	
	<u>Note</u>: The transfer of an aliphatic side chain of a protein from water to a non-polar region causes an energy change of 1000-2000 calories/mole of CH_2 groups (Kauzmann, 1959). The values calculated for van der Waals forces give 400 calories/CH_2 group (Cornwell and Horrocks, 1964). Presumably the two attractions will complement one another.		

London - van der Waals forces	Lipo-proteins - As these forces require satisfactory steric configuration to give close approach they may not be important in natural lipoprotein linkages (Cornwell and Horrocks, 1964). Colloidal adsorption - This is strongly influenced by these forces (de Boer, 1950). (Presumably this will influence the nature of materials adsorbed (e.g. fatty acids, enzymes, etc.). This could be especially true of the interface created by homogenisation. Natural Fat Globule Membrane - During secretion, the fat droplet, as it forms in the cytoplasm, may have a lipid monolayer at its surface containing substantial amounts of lecithin and an adsorbed layer of protein. On approach of this droplet to the plasma membrane at the apex of the cell van der Waals forces may provide the dominant force causing the plasma membrane to attach itself to the protein layer of the droplet (Patton and Fowkes, 1967). Membrane material of homogenised	Surface active agents. (Acting to increase hydration these will reduce zeta potential).

	artificial preparations - Van der Waals forces have been proposed to explain the binding force creating complexes that are found after homogenisation of mineral oil into skim milk (Fox et al, 1960). The results show that milk proteins can be adsorbed onto a simple hydrocarbon substrate but this does not eliminate the possibility of hydrophobic bonding which is not separately considered by these authors.	

Consideration of the complexity of the materials that are to be solubilised in the various membrane leads to the conclusions first, that previous workers have achieved incomplete success at characterising fat globule membrane material because complete solubilisation has proven unachievable, and secondly, that adequate solubilisation will require a number of solubilising agents that act so as to be complementary to one another rather than dependence on just one agent.

In view of these observations a series of experiments were devised to find a technique that would give the best overall solubilisation of the material.

INVESTIGATION OF SOLUBILITY PROPERTIES OF MEMBRANE MATERIALS

Section 8.1

1. The Influence of Various Dispersants upon the Dispersion of Membrane Material from Homogenised Milk -

As the aim of the present study was to investigate the material adsorbed onto the fat interface as a result of the homogenisation process, a study aimed at the maximum solubilisation of membrane material of this origin was chosen rather than a comparative study of the solubilities of membranes of various origins.

Procedure:

200 mg of a membrane material, isolated from the separable fraction of globules from homogenised milk was triturated in a glass dish with about 2 mls of a solution of dispersant. The slurried material was washed into a test tube and made up to 10 ml with dispersant solution and shaken for four hours to permit hydration of protein material. Half of the resultant dispersion was analysed for nitrogen content. The other half was pre-filtered through a No.2 sintered glass filter (stated to have a maximum pore diameter of 40 - 50 μM) to remove completely undissociated material, and subsequently through a No.4 sintered glass filter (stated to have a maximum pore diameter of 5 - 10 μM). The final filtrate which contained only that material which had a particle diameter of below 10 mM was analysed for nitrogen content. Because sintered glass filters vary in uniformity of pore structure the same No.4 filter was used for all experiments. The concentration of nitrogen in the filtrate was then related to the original value as measured by concentration of nitrogen in the unfiltered material to give a measure of solubility. The measure is arbitrary and usable only for comparative purposes where conditions of experiment are held steady while changing only the dispersant.

Note 1: No attempt is made to relate nitrogen level to protein

level for membrane materials are diverse in nature and a constant value such as 6.38 would infer unreasonably that natural protein is being measured.

Note 2: Where EDTA was used as dispersant its nitrogen content was either calculated or measured from "blank" values and subtracted from the values determined for it was assumed to be completely soluble and so bias the results by giving disproportionately high levels of nitrogen in the filtrate.

Note 3: Nitrogen determination. The determination of nitrogen at low levels (perhaps 50 - 100 mg. N in the final sample) imposed difficulties. In gener 1 the technique described by the New Zealand Standard Methods for Chemical Analysis of Liquid Milk and Cream (N.Z.S. 2246 : 1969) was followed but modified to suit micro distillation procedure. (For details see Appendix 2). The precision of titrations from duplicate distillation of the digested materials was not always satisfactory however. It was learned that use of sodium thiosulphate to release ammonia in the distillation step may on occasions lead to production of H_2S gas which may be absorbed into the boric acid solution and interfere with subsequent titration. The alternative use of sodium hypophosphite proved more satisfactory. Results of determinations on the cream powders gave the following differences in titration, with 0.02 N HCl between duplicate distillations: 0.03 ml, 0.07 ml, 0.03 ml, 0.01 ml, 0.04 ml, 0.03 ml, 0.05 ml, 0.00 ml, 0.01 ml. Such differences are within the error caused by measurement of the liquid through the burette and in detecting the exact endpoint of the indicator. Consequently sodium hypophosphite was used instead of sodium

thiosulphate for some of the nitrogen determinations related to the washing procedure (Section 6) and all of the following investigations.

Results

Table 4: Influence of Dispersants

Treatment for dispersion of membrane material from homogenised milk.	% N passing the No. 4 filter (max. pore size 5 - 10 μ)
1 Dispersed in 1M. KCl	44
2 Dispersed in 1M KCl + 1 Drop Teepol detergent + 0.01 gm EDTA (Disodium salt of ethylene diamine tetra acetic acid).	53.5
3 Dispersed in 3 drops Teepol detergent	38
4 Dispersed in 3 drops Teepol + .01 gm EDTA	62.7%
5 Dispersed in 1% Sodium Dodecyl Sulphate (S.D.S.) + .01 gm EDTA	69.6%
6 5M Guanadine hydrochloride + 2-mercaptoethanol	No result
7 30% Alcohol	No result - protein floculated.

Other dispersants were tried but found unsuitable for various reasons. 5M guanidine hydrochloride plus 2-mercapto-ethanol may have been satisfactory but could not be satisfactorily analysed by Kjeldahl procedure for nitrogen recovery from the guanidine group is not complete (Bradstreet, 1965). The use of KCl which will dissociate glycoprotein from natural fat globule membrane (Swope, Rhee and Brunner, 1968) is unsuitable because the resultant solubilised material would have too high an ionic strength for satisfactory application to DEAE cellulose chromatography or electrophoresis. 2-Mercaptoethanol alone was of no apparent value on inspection of the dispersion which confirms an earlier report (Harwalkar and Brunner, 1960). In the absence of detergent the membrane

materials were not suitably wetted by the dispersants so that some trials carried out without detergent inclusion proved meaningless.

Discussion - The results show clearly that detergent has a considerable value in improving dispersion. This is in accordance with published results (Harwalkar and Brunner, 1965? 1960) and was later shown to be due not only to its effect on wetting the material but also to a specific reaction with the material. It is not of very great value when used alone however.

The value of EDTA in combination with detergent (necessary to assist wetting) is notable and has been previously reported as a peculiarity of the casein-lipid complexes of homogenised and pasteurised milks (Cerbulis, 1969). Its value in dispersing the casein complex is also known (Thompson, Kiddy, Johnston and Weinburg, 1964). Possibly this indicates the presence of casein in the membrane material of homogenised milk and additionally it may indicate that EDTA assists dispersion of natural membrane as suggested above or even that the protein-lipid complex is stabilised by ions that are chelated by EDTA.

Section 8.2

The Influence of Heat upon the Dispersion of Membrane Materials from Homogenised Milk

Procedure:

The procedure of experiment was similar to that described in Part 1 except that after the shaking step the dispersion was heated prior to filtering.

Results:

Table 5:

Material Used	System of Dispersion	% N passing the No. 4 filter
200 mg of a freshly prepared membrane preparation from the separable globules of homogenised milk.	6) Add 1% SDS, + 0.01 gm EDTA, shake, boil, cool, filter.	67.4
	7) As above but filtered hot (about 90°)	96.2
	8) As for 7 but heated and filtered at 50°	73.5
Membrane material from the smallest globules of homogenised milk (minor fraction)	9) As for 7	74.0

Discussion: In this series of results the extent of solubilisation by use of detergent and EDTA is confirmed while using membrane material prepared separately from a different milk. The material, when heated and cooled before filtering (Expt. 6.) shows the same solubility as a non-heated sample (Expt. 5.) but if the material is heated and filtered hot (Expt. 7.) the proportion that can pass the filter is improved. Unfortunately filtering while hot has a number of drawbacks. First, the minor fraction does not appear to be so well dispersed at this temperature (Expt. 9.); secondly, high temperatures will lead to denaturation of enzymes and possibly other membrane components (as may be indicated by the low value of Expt. 9.); and thirdly, the use of vacuum to draw the dispersion through the filter while it is hot and contains detergent leads to frothing which might further denature the solubilised protein materials. It is shown that heating and filtering to 50° will still improve the extent of solubilisation with little damage to enzymes or other membrane components. To avoid the frothing problem centrifugal separation of insoluble

materials proved satisfactory.

Centrifugation also provides an indication of the reason why heat is of value for not only does a sediment of insoluble material appear at the base of the tube but also a disc of solid white fat appears at the top. As previously stated (Section 7) the butanol and acetone extracted membrane powders contain a proportion of fats that are both acetone insoluble and of high melting point (above 40°). These fats have been previously recognised as being associated with the natural membrane but are probably not an integral part of it (Vasic and De Man, 1966; Mattson, Swartling and Nilsson, 1969). In the acetone dried powders they appear to provide a matrix in which the protein particles are imbedded and thus the protein cannot be wetted by the solubilising agent. On melting the fats, particles of acetone powder can be easily seen to disintegrate and dispersion of the protein by the reagent can then proceed.

This provides suitable explanation for the above results for while fat is in the solid state it blocks the pores of the filter and so reduces the pore size of the filter so that less protein material can pass. This is indicated too by the fact that ether can effectively clean the filter. Possibly also the influence of heat may be in part to assist the action of the detergent. Assuming that these act primarily through the formation of hydrophobic linkages with protein side chains and also increase the order of the protein molecules (Tanford, 1968) heat will tend to support this action for the transfer of an aliphatic side chain from water to a non-polar region in a protein is endothermic to the extent of 1000 - 2000 cal per mole of CH_2 groups (Kauzmann, 1959). Raised temperature also improves detergent activity by altering the nature of the micelles formed (Elworthy, Florence and McFarlane, 1968).

Having established the value of heat in improving solubilisation a number of restraints are placed upon the way in which other solubilising agents can be used. It is not possible to heat proteins in the presence of urea because heat increases the likelihood of carbamylation of amino and sulphydryl groups of proteins by the cyanate that forms as a breakdown product of urea (Stark, Stein and Moore, 1960). Furthermore the use of 2-mercaptoethanol prior to heating is excluded for it is highly volatile and would be lost. These two reagents can, however, be added after the heating stage when required for subsequent analytical procedures.

Section 8.3 The Technique of Solubilisation of Membrane Materials using Sodium Dodecyl Sulphate

In light of the above investigations the following solubilisation technique was adopted.

300 mg of membrane material, 100 mg of S.D.S., and 10 mg of EDTA were triturated with the buffer solution that was appropriate to the particular analytical procedure in use. Volume was made up to 10 ml with buffer and the suspension was shaken for 2 - 4 hours. It may have then been held for up to 20 hours at 5° according to convenience. The suspension was warmed to 50°, then while still hot centrifuged at 4000 r.p.m. for 5 minutes in a BTL bench centrifuge. This gave a firm pellicle of fat and a sediment of insoluble material. 5 - 8 mls of the central cloudy liquid layer was withdrawn by pipette and urea was added to make the solution 4.6 Molar with respect to urea. This solution was held for not more than 2 hours. Two drops of 2-mercaptoethanol were added just prior to beginning the analytical procedures.

Conclusion

Consistent with the conclusion that as a number of bonding

forces are to be broken a number of reagents will be necessary the solubilisation technique employs sodium dodecyl sulphate (S.D.S.) to wet the material, to break hydrophobic bonds and weaken van der Waals forces, di-sodium ethylene diamine tetra acetic acid (EDTA) to chelate calcium and magnesium, urea to disperse hydrogen bonds and weaken hydrophobic bonds, and 2-mercaptoethanol to break disulphide linkages. These last two reagents are employed after centrifugation so do not react with the "insoluble" fraction. In addition the solution is warmed to 50° to melt adherrant fat and buffer pHs are between 9 and 11 and this assists the action of EDTA and assists protein solution.

Section 8.4

The Use of Non-ionic Detergents as Solubilisation Agents:

Because of its ionic nature S.D.S. affects the results of experiments by obscuring the native charge of protein materials. To gain information relating to this property the use of non-ionic detergent to serve the same functions as S.D.S. in the solubilisation procedure was investigated. The non-ionic detergent Amidox 10L (an ethoxylated alkyloamide) has been shown to be of limited value in dispersion of natural fat globule membrane (Harwalkar and Brunner, 1960).

Treatment of fat globules with sodium deoxycholate results in the desorption of lipo-protein particles which account for 45% of the total weight of the natural fat globule membrane. The mechanism of release of such particles is unexplained but lack of damage due to the detergent is indicated because similar particles released from the membrane by physical (churning) procedures have similar properties. The particles exhibit solubility in water (which could be contributed to by bound deoxycholate), ultra-centrifuge sedimentation characteristics, precipitation characteristics with changes in pH and with ammonium sulphate concentration, which indicate that

reaction with detergent has not resulted in serious modification of their native properties. Similarly, the activities of alkaline phosphatase and xanthine oxidase suffer little reduction after reaction with the detergent (Hayashi and Smith, 1965). Lipo-proteins that have been isolated from the fat globule membrane and treated with 2% sodium deoxycholate (or a number of other lipo-protein solvents) cannot be characterised by electrophoresis however (Alexander and Lusena, 1961).

The ability of non-ionic detergents to solubilise cell membranes has been demonstrated with bacterial membranes and with human red corpuscles (Elworthy, Florence and McFarlane, 1968). In the latter case Triton X-100 (polyoxyethylene iso octyl phenol) was used and although extent of solubilisation does not appear to be great it did lead to the release of membrane components without loss of biological integrity (Bonsall and Hunt, 1966). The membrane of cerebral microsomes has also been treated with the non-ionic detergent series "Lubrol" (a polyoxyethylene ether series) to give solubilisation of up to 50% of the protein (Swanson, Bradford and McIlwain, 1964).

Solubilisation techniques when using non-ionic detergents depend largely upon the extent of hydration achieved and it is known that such detergents complement one-another's activities. The above observations appear to have been carried out using single chemicals only. One of the important properties of non-ionic detergents that can be used to assist the choice of an appropriate blend of detergents is the hydrophile-lipophile balance (HLB). The HLB value is an expression of the relative simultaneous attraction of an emulsifier for water and for oil and relates primarily to the proportions of hydrophobic and hydrophilic components of the molecules. For every material

to be solubilised emulsifying agents are chosen to provide an appropriate HLB value. The HLB of a single emulsifier can be found by consideration of its formula (or experimentally) and the HLB value for a mixture of two emulsifiers can be calculated readily (Griffin, 1965).

Modifying the application of the HLB system however are the differing modes of action of the various emulsifiers for which various models have been proposed (Nakagawa, 1967). For example, ethylene oxide derivatives have the property of hydrogen bond formation between the ether oxide and water thus creating a high degree of hydration about the solubilised material but the solubilisation effected reaches a peak value, instead of increasing, as length of polyoxyethylene chain is increased (Sherman, 1968; Nagakawa, 1967). The same observation applies to the solubilisation of microsome membrane (Swainson, Bradford and McIlwain, 1964).

The numerous factors that influence efficiency of solubilisation have been recently reviewed (Elworthy, Florence and MacFarlane, 1968).

To solubilise the various membrane materials for the present study it was necessary to choose an appropriate pair of emulsifiers. The choice was made after the following considerations: (1) As the materials are strongly hydrophobic the use of a polyoxyethylene derivative having good hydration properties was indicated. It is known that the most effective surfactants of this series contain a relatively short number of ethylene oxide units, n. e.g. (N = 20) (Elworthy et al, 1968; Swanson et al, 1964).

(2) In combination, sorbitol improves polyoxyethylene detergents.

(3) Emulsifiers with side chains that are

sterically different form mixed micelles that do not pack tightly and so inclusion of solubilisate into the micelles is facilitated (Elworthy et al, 1968; Nagakawa, 1967).

(4) So that a wide range of HLB values could be tested it was convenient to choose two compounds having widely different HLB values.

On these bases the two emulsifiers chosen were:
Tween 20 (polyoxyethylene 20 sorbitan monolaurate, HLB 16.7) and Span 85 (sorbitan trioleate, HLB 1.8) as supplied by Atlas Chemical Industries Inc.

Procedure for determination of appropriate HLB value:

Membrane material isolated from separable globules of homogenised milk and from natural milk fat globules were treated. The emulsifying agents Tween 20 and Span 85 were diluted 1:10 and mixed in various proportions to give a range of HLB values accordingly to the relationship:

(HLB of mixture = xA + (1 - x)B)

where x is the proportion of the surfactant having an HLB of A whilst the other surfactant has an HLB of B.

300 mg of membrane material and 10 mg of EDTA were mixed with 1 ml of the diluted emulsifier, triturated and made up to 10 ml with water. (This gave a 1% concentration of emulsifier which exceeded cloud point in all cases). The rest of the investigation was carried out as for the investigation of other dispersants except that the dispersion was cleared after heating to 50° by centrifuging at 6000 r.p.m. for 5 minutes instead of filtering on a coarse filter. A No. 4 sintered glass filter was again used for the final removal of material insoluble at 50°.

Results: The results are presented in Figure C.

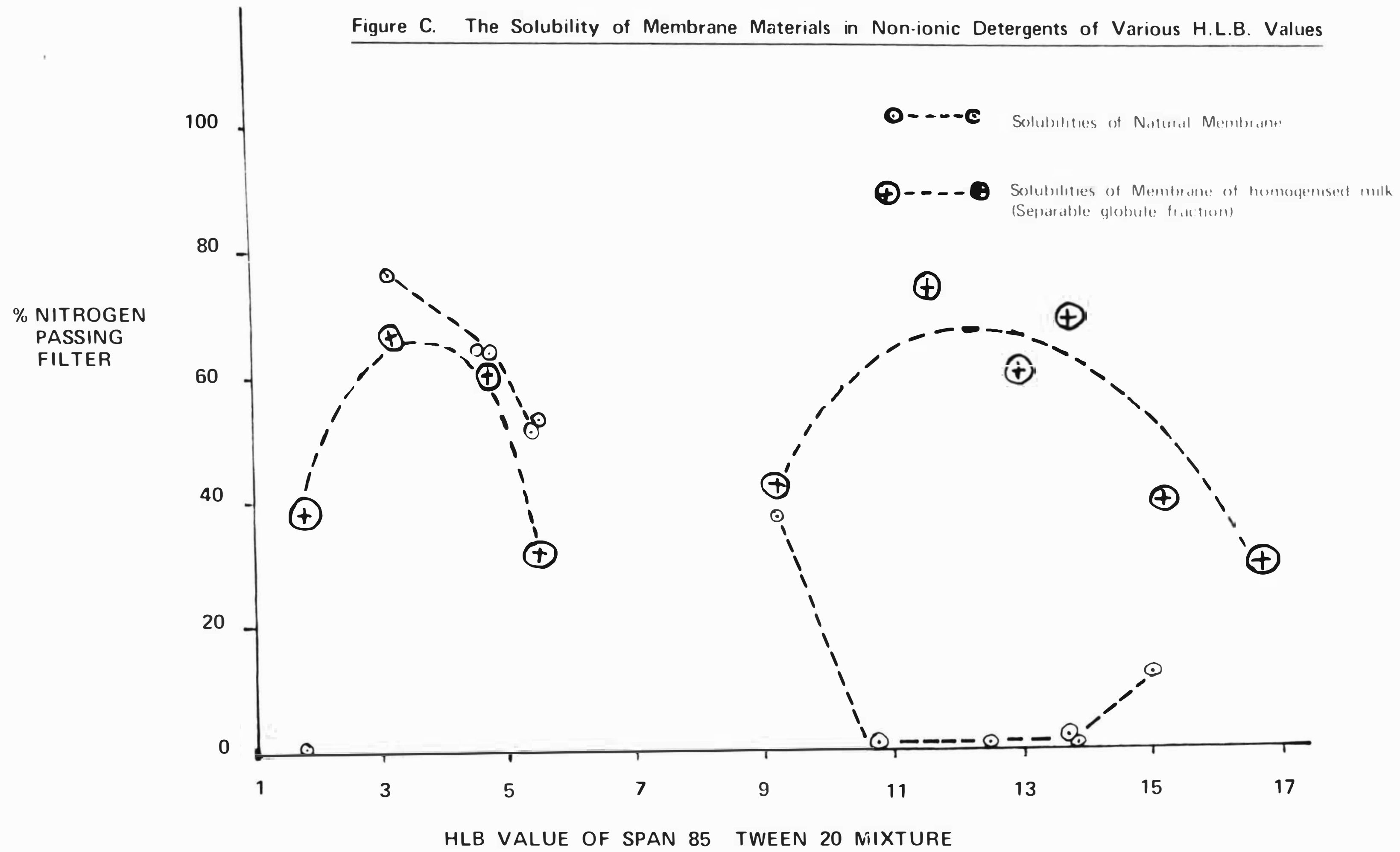


Figure C. The Solubility of Membrane Materials in Non-ionic Detergents of Various H.L.B. Values

Also, for comparative purposes the emulsifiers, Tween 60 (polyoxyethylene sorbitan mono stearate, HLB 14.9) and Span 60 (sorbitan monostearate HLB 4.7) were investigated at HLB levels between 12.8 and 14.9 but found to give no solubilisation of the membrane material isolated from the separable globules of homogenised milk.

Discussion:

The results in Figure C show that the effectiveness of the non-ionic detergents is dependent upon a number of factors. These are:

(1) The HLB Characteristics of the detergent mixture

Solubilisation of the material at the most appropriate HLB values is as effective as that achieved with sodium dodecyl sulphate. To this extent a major aim of the investigation has been achieved. At other HLB values solubilisation is poor or not achieved at all. The S.D.S. molecule does not fit well into the HLB concept (though it can be assigned a nominal value of 40) because its primary difference from the other emulsifiers is its charged nature. From the results, however, it is apparent that the reaction of the membrane material with the non-ionic emulsifier is adequate to provide 70% solubilisation without a contribution from the charged group. These results indicate that the charged group of S.D.S. need take no direct part in the association with the membrane material.

In the HLB range of 3 - 6 emulsifiers act to assist water in oil emulsion (Becker, 1967) and are highly hydrophobic in character. This apparently suits the natural membrane. It may be for this reason that butterfat emulsification in recombined milk plants is most satisfactorily achieved by use of glycerol mono stearate which had an HLB value of 3.8. Such emulsifiers are not readily dispersible in water which

indicates that the membrane material itself must provide the necessary hydrophillic groupings to form the oil in water emulsion of this experiment.

In the HLB range 7 - 9 emulsifiers act as wetting agents. While this action is of some value this cannot be the only function served by the emulsifiers.

In the HLB range 8 - 15 oil in water emulsions form and the emulsifier acts to provide sufficient hydrophobic groups to bind with the solubilisate material and a high proportion of hydrophillic groups to stabilise it in water suspension. This was of no value to the natural membrane but was important to a component of the membrane from homogenised milk.

(2) The Type of Hydrophobic chain of the detergent

Lauric acid is the hydrophobic component of both successful detergents (S.D.S. and Tween 20) but not in the unsuccessful ones (Tween 60 and Span 60). But further, the successful detergent contains a mixture of fatty acids (oleic and lauric) which would have different steric characteristics and so form an open micelle that would readily incorporate solubilisate. The unsuccessful mixture contains only stearic acid and would form tightly packed micelles less suited to solubilisation.

The importance of using a blend of non-ionic detergents is established from the foregoing considerations and this may explain why previous reports, in which only single emulsifiers were employed, show only partial success in solubilisation.

(3) The Nature of the Solubilisate - the membrane material

The results show important differences between the reactions of membranes from normal and homogenised milks. Both

are solubilised by emulsifier blends having hydrophobic characteristics but the material from homogenised milk apparently has an extra component which is best solubilised by emulsifier blends having hydrophillic characteristics. This can be explained by assuming that both materials contain material of the natural membrane but homogenisation has caused an additional material to be adsorbed. Such material may consist of a number of fractions having varying HLB requirements. As more than 50% of the nitrogen is soluble at low HLB values this additional adsorbed material must have some solubility with hydrophobic emulsifiers also.

From consideration of the mechanism of emulsifier action it can be postulated that the natural membrane material has an adequate complement of hydrophillic groups to stabilise it in water suspension and the function of the emulsifier is to satisfy exposed hydrophobic groups by complexing them with its own hydrophobic groups. The adsorbed material of the homogenised milk on the other hand is possibly hydrophobic in nature requiring an emulsifier with excellent properties of water dispersibility to hydrate it and stabilise its suspension in water.

Conclusion

The results indicate a means of partially fractionating the membrane material of homogenised milk. By use of an emulsifier mixture having an HLB of 11.6, material presumed to have been adsorbed due to homogenisation, is readily soluble whereas native membrane is not.

Consequently membrane materials were solubilised by the following technique:

300 mg of the membrane material was triturated with the buffer appropriate to the particular study, 0.01 gm EDTA,

0.33 ml of a 1:10 dilution of Span 85 and 0.66 ml of a 1:10 dilution of Tween 20. The procedure then followed that detailed for solubilisation with S.D.S. as the reagent.

Section 8.5 The effect of Lipid Extraction and Solubilisation upon the Membrane Components

The preparation of solubilised membrane material includes washing, freeze-drying, fat extraction, and heating to 50° and it is possible that progressive alteration of the original materials could occur. Evaluation of the extent of such changes cannot be directly made for there is no "standard" membrane material with which to compare. One indicator of membrane stability is the enzyme alkaline phosphatase the beta form of which is present in the natural fat globule membrane (Peereboom, 1968) and which should remain active in the solubilised material. This enzyme is fairly heat stable and would not be destroyed at 50° (Jenness and Patton, 1959) though a small extent of denaturation could occur. Simple detection of the active enzyme would indicate that severe denaturation due to laboratory procedures has not occurred. Because of the possible direct effect of detergent on the substrate, detergent solubilisation was not studied in this manner.

Procedure:

0.02 grams of membrane material (approximately equivalent to the weight of membrane in a 10 ml sample of milk having 4% fat) was added to 10 ml of carbonate/bicarbonate buffer, warmed to 50° for 20 seconds and cooled rapidly under a stream of water to air temperature. The phosphatase thus extracted was then determined from its action on the substrate disodium para nitro phenyl phosphate according to the technique of Aschaffenburg and Mullen. Each type of material was available from three separate milks.

Results:

Table 7.

Origin of Membrane Materials	Enzyme Activity (expressed as micro-grams of p-nitro phenol released per ml of suspension in 2 hours).	
	Freshly extracted materials	Material after 1 month storage at -15°
Homogenised milk		
- minor fraction	25, 42, 42	18
- separable fraction	25, 25, 18	0
Non-homogenised milk	10, 42, 10	0

Discussion:

The results clearly show that the alkaline phosphatase has been retained from the original milks with varying success but without total denaturation. The actual levels of enzyme activity in the various preparations cannot be used to support conclusions about the preparations because they may merely reflect variations in composition of the membrane preparations. A constant weight of material taken does not necessarily reflect a constant weight of membrane from the original milks and efficient enzyme extraction from the materials is not very likely because dispersants were not used. Extracts were prepared in small quantities because of the difficulty in preparation and compositonal analyses were not attempted.

After a month's storage of the membrane materials at -15° however the enzyme has lost its activity and this indicates that materials should be freshly extracted before analytical procedures are performed. The enzyme appeared to be stable

in cream powders for it could be extracted after 2 years storage at -15°. This indicates that reactions can occur within the powdered, fat extracted materials during storage at -15° and it was further observed that insolubility of samples or anomalous results during electrophoresis or chromatography could be corrected by preparation of fresh membrane materials. Similarly, reactivity leading to insolubility within 3 days at 5° has been reported for proteo-lipid material that had been fat extracted and powdered (Tenenbaum and Folch, 1966).

The results also show that homogenisation has not led to phosphatase inactivation. Fat globules of the size recovered in the "minor" fraction did not occur in the original milk yet forces which have been sufficient to cause adsorption of protein material from the serum onto these globules has not prevented active enzyme from being either retained or freshly adsorbed from the serum along with the other materials.

Conclusions

Studies of the solubility characteristics of the membrane materials support the following conclusions:

1) EDTA is of value in improving the dispersion of the membrane material from homogenised milk. This could arise from some combination of at least three factors. Remnant natural membrane material may still be present and be structurally dependent upon ions that can be chelated by this dispersant. Casein, which is known to be dispersed by EDTA may be present on the newly formed membrane. The formation of fat/protein links may be structurally dependent upon ions that can be chelated by EDTA.

2) Detergents are of value in dispersion of the materials and can act through wetting the material, breaking van der Waals attractions, breaking hydrophobic bonds, or some combination

of the three. Because non-ionic detergents, at the proper HLB value, are apparently as effective as sodium dodecyl sulphate it is concluded that charge is not of vital importance though this is not to say ~~that it is not to say~~ that it has no value.

3) Heat is of value in dispersing the material. It acts to liquify fatty materials present and improve the emulsifying action of the detergents.

4) A combination of dispersion agents is desirable to disperse the numerous bond types present in the complex material.

5) As the membrane of homogenised milk is soluble at two HLB values it may contain two components; one adsorbed and one remaining from the original membrane. If so the adsorbed material is probably hydrophobic in character.

6) On the basis of relative solubility the adsorbed material may be solubilised preferentially to the remnant of the natural material by use of non-ionic detergents having an HLB of 11.6 and containing both lauric and oleic acid residues.

7) After the extraction process it is possible to detect active alkaline phosphatase in all membrane preparations so the procedures cannot be severely denaturing. The presence of this enzyme on the membrane of the smallest globules of homogenised milk indicates either that it is not destroyed by the process or that it can be adsorbed from the serum subsequently to the homogenisation.

Section 9.

Characterisation of the Protein Materials isolated from the Various Membranes

Characterisation of the protein materials was performed by two principal techniques. The material that had been solubilised with ionic and with non-ionic detergents was fractionated by use of DEAE (di ethyl amino ethyl) cellulose chromatography and the fractions were checked for purity by scanning at ultraviolet wavelengths. Also electrophoresis in polyacrylamide gel was used to separate the proteins of the membrane materials and of the fractions.

These procedures provide information about two fairly distinct properties of the proteins; the charge on the protein molecules is the principal property distinguished by the chromatography and the charge as modified by molecular size and shape is the principal property distinguished by polyacrylamide electrophoresis.

9.1 Column Chromatography on DEAE Cellulose

The principle of separation by column chromatography is that multiple electrostatic bonds form between charged sites on the adsorbent and sites bearing opposite charge on the surface of the protein molecule. The number of such bonds that can be established will determine the concentration of competing ions required for release of the bound molecule. The extent of bonding established depends upon the protein's charge density, the protein's charge distribution (where it affects the availability of charges to be adsorbed). On occasion non-electrostatic forces may play a part.

Proteins, adsorbed onto granules of the DEAE cellulose, are released by a buffered eluate containing progressively increasing levels of sodium chloride. The least strongly

bound proteins desorb at low sodium chloride concentration and the more strongly bound proteins are released progressively as the sodium chloride concentration is increased. The released proteins then elute with the flow of buffer and can be measured and collected for further investigation. The general technique has been well described by Peterson and Sober (1962).

The technique does not appear to have been previously used in study of membrane materials though ion-exchange resin chromatography in the detergent Triton X-100 has been used partly successfully to purify the membrane protein of E. coli (Kennedy, 1969). The specific technique employed in the present study has been successfully used to separate and characterise caseins (Thompson and Kiddy, 1964; Thompson, 1966). It was assumed that such a technique should be satisfactory because casein and/or other milk proteins should be adsorbed onto the fat/serum interface as a result of homogenisation of whole milk and these proteins, must be the origin of the adsorbed membranes of the artificial preparations. It was further assumed that such materials, once solubilised would show adsorption properties on DEAE cellulose bearing similarity to the original proteins. This assumption appears to have been made also in interpreting the free boundary electrophoretic patterns of "alpha-casein" and "beta-casein" on membrane material of homogenised milks (Jackson and Brunner, 1960).

Since the present study was initiated a technique for quantitative determination of casein components on DEAE cellulose after alkylation of the protein has been described (Rose, Davies and Yaguchi, 1969). Alkylation was not necessary to give satisfactory resolution of the casein components but quantitative studies of the components using 2-mercaptoethanol with un-alkylated samples were insufficiently precise, for small peaks of material that

desorbed at low sodium chloride levels appeared and interfered with the results. The system used in the present study includes the use of 2-mercaptoethanol directly and the interpretation of results must allow for this observation. The same equipment and materials as used in the present study have been employed to give near quantitative information about the variation of casein components during the milk production season (Sanderson, 1970).

Procedure

Materials were prepared as follows:

Buffer was prepared in bulk and contained 0.01 M imidazole and 4.5 M urea and was adjusted to pH 7.0.

Urea solutions were made up to 6 Molar, filtered to remove impurities, and passed through a cake of fine DEAE cellulose on a buchner funnel. DEAE cellulose (Whatman DE 52 microgranular, pre-swollen) was prepared as stipulated by the manufacturers.

The chromatography was performed as follows:

Before each use the cellulose material was mixed in bulk with buffer and allowed to settle, the buffer decanted, and fresh buffer added. This procedure was repeated several times to remove all protein material and sodium chloride that could remain from earlier use. The slurry of cellulose was then run into a Kontes chromaflex column (20 mm diameter x 300 mm length) and packed by pumping buffer onto it for 2 hours using a Sigma peristaltic pump.

The prepared sample containing 300 mg of membrane material, freshly mixed with 2-mercaptoethanol, was pumped onto the top of the column. This was followed by buffer pumped at the rate of 35 ml/hour from a 2 chamber gradient device (500 ml buffer and 2 drops 2-mercaptoethanol; 500 ml buffer, 2 drops 2-mercaptoethanol and sodium chloride made up to 2 molar) which fed a linearly increasing concentration of sodium chloride on to the column. Chromatography was carried out at room temperature for about 16 hours during which time the sodium chloride concentration in the eluant rose to about

1.2 Molar. The eluted protein solution was monitored at 280 nM by passing through a Unicam (SP 500 series 2) spectrophotometer equipped with four flow-through cells of varying pathlength (1, 2, 5 and 10 mm). The spectrophotometer was programmed to read every 3 minutes and was coupled to a Sargent recorder (model SRLG) set to record, optical densities between 0 and 1. The eluate then passed to a Gilson sequential fraction collector, set to collect 5 ml samples. This procedure provided a graph of optical density at 280 nM against time and an event marker identified the points at which the fraction collector moved the tubes.

The liquid containing the fractionated protein was bulked into its fractions and chloroform was added as preservative. Each fraction was dialysed at 4° for 3 days, against four changes of distilled water, to remove buffer. The fractions at this stage had a volume of about 20 - 40 mls (depending on the size of the original peak) and were concentrated to about 5 mls by placing the closed dialysis bag into a small jar, sprinkling polyethylene glycol over it, closing the jar, and holding at 4° for about 14 hours. Water was drawn out of the dialysis bag during this period and the concentrated material, preserved with chloroform, was held in 1 oz. McCartney bottles at 4°.

At a convenient time (always within 1 week) fractions were scanned over the wavelength range 230 - 320 nM in a Beckman DU recording spectrophotometer to indicate purity of the fractions and to indicate which fractions contained protein. Fractions were thereafter held at 4° in the presence of chloroform for electrophoretic investigation.

Discussion

Major differences between membrane materials and casein as analysed by chromatography soon became apparent. Membrane

proteins were not adequately desorbed at a sodium chloride concentration of 0.3 Molar which is satisfactory for casein and consequently the use of a sodium chloride gradient that rose to 1.2 M was introduced. Even at this level there appeared to be protein still adsorbed to the cellulose. This observation must indicate that the materials, as solubilised, are much more highly negatively charged than the native caseins at pH 7.0 (See Section 10.4 for further discussion of this point).

Urea for use in this procedure is subjected to a deionisation step by passing through a bed of DEAE cellulose. A deionisation step is said to improve resolution of protein components and reduce the likelihood of carbamylation of lysine by cyanate ions (Thompson, Gordon, Boswell and Farrell, 1969).

Concentration of the fractionated proteins was performed by osmosis using a high molecular weight anhydrous material outside the dialysis membrane. Polyethylene glycol, a waxy flaky substance with molecular weight of 20,000, was used. This principle has been used for concentration of enzymes and no evidence of damage to the enzymes was detected (Kohn, 1959; Bannatyne, 1969). The technique was simplified by retaining the dialysed protein fractions in the dialysis bag where they remained without possibility of contamination. Re-use of the polyethylene glycol after drying by heating in an oven at 250°F was normally practiced.

Preservation against bacterial action was achieved by holding fractions cold and by addition of chloroform. Toluene was initially used but discontinued because its absorption spectrum interfered with the U.V. scanning of the fractions.

The technique as described contains many steps and is time consuming so that only two or three samples were handled per week. Minor difficulties such as mechanical defects could

ruin an investigation but the process was not, on the whole, difficult to perform. As membrane materials had initially been prepared in triplicate it was not normally necessary to duplicate individual runs though this was occasionally carried out to check the reliability of the technique. Results were required to be qualitative to show the occurrence of differing components in the various materials and did not need to be interpreted quantitatively. Indeed, an artifact peak appeared very early in the elution pattern (see page 75) but this was ignored in studies of casein for it contained no protein and was apparently due to an reaction of 2-mercaptoethanol with some component of the buffer. In the present study the same peak did on occasion contain protein, detectable by electrophoresis and U.V. scanning, so it was never ignored. To this extent the system is not quantitative for the materials that desorb at low sodium chloride concentrations but it is known to be reliable for casein at all peaks after the first.

Section 9.2

Disc Electrophoresis in Polyacrylamide Gel

The value of zone electrophoresis for characterisation of the membrane materials in the present study lies in two main applications. The first is in checking the nature and purity of the fractions separated by chromatography because materials having similar charge but different molecular size can be distinguished. The second is in providing overall patterns for the proteins in the various membrane preparations. The principles of zone electrophoresis in gels and in the presence of urea have been recently described (Poulik, 1966).

Dialysis of the fractionated materials, besides removing buffer, would be likely to remove S.D.S. from protein/S.D.S. complexes. If this occurred to an appreciable extent however presumably the unstabilised protein would precipitate. This did not occur so it appears that the protein/S.D.S. complex remains

after the relatively short dialysis period of three days. Complexes formed between protein and non-ionic detergent blend would not have altered in this fashion because the polyoxyethylene tail with molecular weight of 20,000 would be retained within the dialysis membrane. Subsequent electrophoresis presumably therefore is characterising the protein/detergent complexes and not merely the proteins.

The natural membrane proteins of milk have been previously characterised by electrophoresis and 2 or 3 components in the material that is readily soluble have been detected (Prentice, 1969). The membrane material of homogenised milk has been subjected to free boundary electrophoresis but the value of these results is limited because the resolution achievable with this technique is not great (Brunner, Lillevic, Trout and Duncan, 1953; Jackson and Brunner, 1960). The use of zone electrophoresis on such membrane materials is limited either by lack of membrane solubility or by the fact that natural fat globule membrane proteins may be so large they do not all enter the gel. Starch gel electrophoresis has been found to be unsatisfactory but use of polyacrylamide gels in the presence of detergents is reasonably effective. Even under these conditions a proportion of the protein is too large to enter the gels (Harwalkar and Brunner, 1965).

Despite its potential the use of polyacrylamide gel electrophoresis to characterise detergent solubilised membrane material has not been reported for systematic use in studies of the milk fat globule membrane. S.D.S. solubilised membranes have been extensively investigated by zone electrophoresis in study of erythrocytes (Rosenburg and Guidotti, 1969), ribosomes (Shapiro, Scharff, Maizel and Uhr, 1966), poliovirus (Summers, Maizel and Darnell, 1965) and the bacterium *Escherichia coli* (Vinuela, Algranati and Ochoa, 1967). In all of these cases

the membrane materials are more completely solubilised by detergents than by other dispersants and subsequently give a greater number of electrophoretic bands than can be demonstrated with other dispersion methods. Recovery of 93% of the original membrane protein of adenovirus after S.D.S.-solubilisation and polyacrylamide gel electrophoresis has been demonstrated (Maziel, 1966). It is recognised that such bands represent a protein/S.D.S. complex and to this extent do not represent the original material. For use as a tool in comparative studies of the nature of various membrane preparations this consideration need not be a limitation. That is to say the ability of the materials to form protein/S.D.S. complexes could be a distinctive property of the materials and this property may well be evidenced by the ability of the complexes to migrate differently on polyacrylamide gel electrophoresis.

The use of protein/ S.D.S. complexes to indicate the molecular weight of unknown proteins and polypeptides has been proposed. It has been suggested that the formation of a protein/S.D.S. complex minimises native charge differences between proteins and consequently all proteins migrate as anions of approximately the same charge because they take the dominating charge of the S.D.S. ions. Polyacrylamide gel electrophoresis of the complex should consequently show migration rates which are dependent more upon the retardation of migration by gel pore size than by protein charge. On this basis a relationship between molecular weight and relative migration of the complex has been proposed (Shapiro, Vinuela, Maisel, 1967). This technique has been employed to indicate the M.W. of a protein dissociable from the erythrocyte membrane (Marchesi, Steers, Marchesi and Tillock, 1970) and also for the membrane protein of *Escherichia coli* (Kennedy, 1969). The application of the technique to the fractions separated by DEAE chromatography appeared to be very exciting but, as will be shown (Section 10.4) its reliability is open to doubt.

The electrophoresis of membrane proteins treated with non-ionic detergents has been less widely employed. A number of authors (e.g. Seanu and Oriente, 1961) have quoted with approval an unsupported statement that non-ionic detergents are without effect on proteins (Putnam, 1953). This is not true for lipoproteins, for Triton X-100 alters the blood serum lipoproteins so that their electrophoretic mobility in starch gel is altered. It was considered that the site of attack is the lipid moiety and removal of lipid caused the change of electrophoretic mobility (Scanu and Oriente, 1961). This may not be the complete reason however for non-ionic polyoxethylene detergents also alter the electrophoretic mobility of a number of pure (crystallised) proteins when subjected to paper electrophoresis. The view that treatment with non-ionic detergents results in a greater solvation of the protein molecule has been proposed and this effect alters its electrophoretic properties (Bowsen, Koehler and Barrieux, 1961).

Solubilisation of human red cell membranes by Triton X-100 has been found practicable and gives proteins that respond to electrophoresis. By this technique 12 component protein bands have been detected (Schneiderman, 1965; Schneiderman and Junga, 1968) which compares with 15 - 20 components detected when this material is solubilised with S.D.S. (Rosenburg and Guidotti, 1969).

Consideration of the above information indicates the potential use of polyacrylamide gel electrophoresis of detergent-solubilised fat globule membrane of normal and homogenised milks to characterise them in a manner not previously attempted and to an extent not previously achieved.

Procedure

The equipment used for disc electrophoresis was manufactured at the University and generally follows the plan outlined by

Davis (1964). It was coupled to a Shandon Power Supply. The tris (2 amino - 2 hydroxymethyl propane - 1:3 diol)/Borate/EDTA buffer at pH 9 and the 7% polyacrylamide/4.5 M urea gel as described by Thompson, Kiddy, Johnston and Weinburg (1964) were used. Additionally a large-pore spacer gel containing 3% polyacrylamide as prescribed by Davis (1964) but containing the tris/borate/EDTA buffer was prepared above the small pore gel. Otherwise the technique was that described by Davis (1964).

Samples of whole materials were prepared from recently fat-extracted powders (see Section 8.3). 250 microlitres of this solution, or of the concentrated DEAE fractions, was loaded on to the top of the gel column and 2 microlitres of 2-mercaptoethanol was added to this and a few drops were added to the buffer. Samples were run in duplicate, at least, and were never held in urea for more than 3 hours. With each electrophoretic run a 50 microlitre sample of casein was run as a check reference.

Electrophoresis was carried out at room temperature with a stabilised electric current of 2 m Amp per tube applied for 10 minutes and then increased to 3 m Amp per tube for a further 30, 50 or 80 minutes. Protein staining was performed by soaking the gels in 0.2% amido black in 7% acetic acid for 30 minutes. Decolourisation was achieved by successive washings in 7% acetic acid for 2 - 3 days until clear. Results were recorded by drawing the exact bands that could be seen while still fresh for the clarity of bands decreased after storage in tubes containing 3% acetic acid.

Discussion

The technique described has been used for casein, whey protein, and the various membranes and membrane preparations and gave reasonable success with all. Variation of the conditions of electrophoresis to give clearer separation with any one material might be possible but the aim of the study was

to compare all materials under the same conditions of investigation so some lack of clarity was unavoidable with such a wide variety of protein materials. The DEAE fractionated material commonly gave sharp bands and as these were considered to be the "pure standards" in which protein: protein associations were avoided the conditions were chosen to suit these fractions.

The use of Tris/Borate/EDTA buffer was chosen in preference to the discontinuous Tris/HCl:Tris/Borate buffer of Davis (1964) because it gave definitely clearer bands and a greater number of bands. The use of a discontinuous buffer system has no particular value when using polyacrylamide gels (Poulik, 1966) and furthermore it interfered with separation because a protein component of the natural membrane migrated with the borate boundary and could not be distinguished from the boundary mark. The importance of EDTA in dissociating membrane materials has already been noted and apparently this is true during electrophoresis also. The value of EDTA in the buffer for casein separation has been established (Thompson et al, 1964).

The time allowed for electrophoresis is commonly established by observation of a band of Brom cresol purple which when added to the samples forms a highly mobile visible band ahead of the protein and electrophoresis can be stopped after this band has travelled a constant distance. Possibly too the migration of protein components can be compared to the migration of the dye to establish a relative mobility figure. In the present study Brom cresol purple formed only a diffuse band when run in the presence of detergent and this practice was not continued. Records have been kept in terms of distance migrated during a set period of electrophoresis and figures appear to be accurate to $\pm$ 0.2 cms when comparing migration distances between gels. Some samples, especially

those carrying S.D.S. ran very rapidly while others, presumably the larger molecules ran very slowly so the time of electrophoresis, which was normally 60 minutes, was varied from 40 - 90 minutes wherever difficulty was found in detecting bands from fractionated materials.

The addition of S.D.S. to the gel mixture has been recommended (Karwlakar and Brunner, 1965) but this was not found to be of value in the present study and it is presumed that the use of EDTA in the system eliminated the need for it in some way. The use of 2-mercaptoethanol in the gel mixture was also found to be unhelpful and it may be that this material moves into the gel with buffer during the electrophoresis.

Attempts were made to stain lipoproteins with fat stains after electrophoresis. Sudan Black in propylene glycol was used for 24 hours and then the gel was decolourised with Xylol and the resultant shrunken gel was reswollen in 3% acetic acid. This system was partially effective in staining material from the natural membrane but it also gave a diffuse orange colour with the detergents which was confusing. It had no value in distinguishing the components of the homogenised material nor the dissociated protein/SDS, fractionated samples and so was eventually discontinued.

Results and Discussion

Section 10 The Effect of Detergents upon Protein Material

To understand and interpret the results of the present study it is first necessary to appreciate the action of detergents upon protein materials and to appreciate the types of complexes that form between protein and detergent and which are to be characterised by chromatography and electrophoresis in this study. Particular interest will be given to the action of sodium dodecyl sulphate (S.D.S.) and non-ionic detergents.

10.1: The Action of Detergents on Proteins

The action of detergents as protein denaturants has been recently discussed in a review (Tanford, 1968 and 1970) in which there is no suggestion that detergents break chemical bonds within proteins but there is evidence of the occurrence of considerable conformational changes. The conclusion drawn is that "proteins are partially unfolded after denaturation by detergents and that existing ordered regions, or newly formed ones, or possibly even isolated hydrophobic side chains can interact with the hydrophobic moieties of the detergent molecules to form micelle-like regions."

The nature of the detergent action appears to be stepwise. Initially a native protein molecule binds a distinct number of detergent molecules one by one and this number is a characteristic of the protein. Beta-lactoglobulin binds 2 or 3 molecules of detergent, probably in a tail-first manner and possibly by van der Waals type of association (Hill and Briggs, 1956). A site for hydrophobic binding which is able to bind butane exists at the surface of beta-lactoglobulin, and is probably a single hydrophobic region (Wishnia and Pinder, 1966). For comparison native bovine serum albumin will bind 10 anions of alkyl sulphate on one or more sets of sites (Reynolds, Herbert, Polet and Steinhardt, 1967).

Different numbers of sites on this protein are available to different ligands and those having longer hydrocarbon tail lengths bind more strongly. Further the binding of neutral ligands is pH dependent (Ray, Reynolds, Polet and Steinhardt, 1966) which presumably indicates that some sites become accessible as protein conformation alters. While the hydrophobic moiety of a number of detergents contributes the major share of the binding, the effect of coulombic forces from anionic detergents does provide a subsidiary contribution to the binding forces (Ray et al., 1966). Initially, however, coulombic forces must be relatively small for the negative detergent ion would have to bind to the protein ion which has a net negative charge. (If the protein has a net positive charge, as at pH levels below iso electric point anionic lipids may well cause precipitation through charge binding, and in such cases non-ionic detergents will provide more satisfactory solubilisation prior to electrophoresis (Braun and Radin, 1969).

As the number of detergent molecules reacting is increased the protein molecule begins to open up so that new binding sites become accessible and changes in the properties of the sites become observable as small specific viscosity changes and extent of such opening up varies with the type of detergent and the type of protein. The driving force for any degree of unfolding may be one or a combination of the following:

(a) electrostatic repulsion between charges of the bound detergent ions.

(b) penetration of the hydrocarbon tail of the detergent into the apolar regions of the protein, and

(c) binding-induced changes in the protein:hydrogen ion equilibrium (e.g. a change in the iso-ionic point of the detergent:protein complex) resulting in an increase in electrostatic repulsion between charged parts of the native protein and so altering the protein structure

(Reynolds et al, 1967).

Some of the points of attachment appear to be in the region of the hydrophobic amino acids tyrosine and tryptophane (Bigelow and Sonnenburg, 1962) and lysine has also been implicated as a site of reaction (Markus, Love and Wissler, 1964). Such disturbances of the hydrophobic areas of the protein alters the conformation so as to increase the extent of formation of alpha helical regions within the proteins (Tanford, 1968). In beta-casein which has a disordered structure in the native state, reaction with detergent creates about 20 - 40% of alpha helical regions (Jirgensons, 1967).

This progressive conformational change is accompanied by highly co-operative binding of detergent molecules because simultaneously and/or subsequently a third type of reaction occurs. In this the detergent molecules, already bound to the protein possibly associate with detergent in solution in a manner analogous to that of micelle formation (Hill and Biggs, 1956).

Possibly the detergent grows around the binding site on a protein backbone to form a micelle whose size is limited by the chemical and physical properties of the detergent, by the ionic environment, and by steric hindrance. The end result might be that there are beads of micelles strung along the protein backbone (Pitt-Rivers and Impiombato, 1968). An alternative possibility for micelle structure is that the molecule rolls up and is surrounded by detergent (McKenzie, 1971).

10.2: The Conditions that influence Detergent Action upon Proteins

(a) Concentration

Detergents are distinctive as denaturants in that they react at very low concentrations normally well below 1% and

reactions are commonly completed below or near the critical micelle concentration (Tanford, 1968). To illustrate this point, the critical micelle concentration for sodium dodecyl sulphate is $8 - 9 \times 10^{-3}$ Molar at temperatures between 20 and 40°C (Ogipow, 1964). The 1% solution used in the present study is equivalent to 3×10^{-2} Molar so complete reaction is to be expected.

(b) <u>The Presence of Disulphide Linkages</u>

Breaking of disulphide linkages apparently opens out the protein so as to expose new binding sites and reduction with appropriate agents in the presence of detergents will result in an increase of the amount of detergent bound to proteins which contain disulphide linkages (Pitt-Rivers and Impiombato, 1968; Cheeseman and Jeffcoat, 1970). In the present study mercaptoethanol serves this function.

(c) <u>Time of Reaction</u>

Elegant studies of the reaction of S.D.S. with pure proteins have been performed by permitting the S.D.S. in solution to dialyse into the protein suspension while it is held in a dialysis sac. Under such systems 16 hours was considered adequate for reaction with a range of proteins (Shapiro, Vinuela, and Maizel, 1967) and 48 hours was considered adequate for reaction with casein (Cheeseman and Jeffcoat, 1970) but for other proteins equilibrium was reached after reaction times varying from 4 to 10 days at room temperature (Pitt-Rivers and Impiombato, 1968). In the present study the materials were shaken directly with S.D.S. at concentrations in excess of the necessary level for about 4 hours at room temperature.

(d) <u>Temperature</u>

It is a general property of detergents that for a number of physical reasons solubilisation is improved as temperature is raised (Nakagawa, 1967). Excessive temperatures may harm proteins but use of 50° in the present study is not likely to

do so and is employed for reasons other than improving the detergent effect as will be discussed later.

(e) Reversibility of Detergent:Protein complexes

The S.D.S.:Protein complexes are dissociated by exhaustive dialysis and the properties of the protein may return to normal (Hill and Briggs, 1956). Proteins that are not stable in the absence of S.D.S. will, however, precipitate as S.D.S. is removed from the complex (Pitt-Rivers and Impiombato, 1968).

In the present study fractions isolated from membrane materials by chromatography were dialysed against distilled water for 3 days at 4°. The membrane proteins are unlikely to be stable in the absence of S.D.S. because they solubilise poorly in its absence and, as precipitation occurred rarely, it may be assumed that this treatment did not cause complete dissociation of the S.D.S.:Protein complexes. The function of the dialysis step was therefore to remove buffer prior to concentration and storage.

In the case of the non-ionic detergent:protein complexes, precipitation was not observed after dialysis so the same conclusion may be drawn. Presumably also the large poly-oxyethylene molecules (MW = 20,000) would not pass through the membrane of the dialysis sac. The nature of the complex formed between detergent and membrane proteins has been assumed to be comparable with the complexes that form with proteins. The technique of S.D.S. solubilisation has been widely used for dissociating biological membrane proteins prior to electro-phoresis as has already been discussed (see Section 9.2 - Disc Electrophoresis on Polyacrylamide Gel).

10.3: The Action of Detergents upon Membrane Proteins

Reaction of S.D.S. with the erythrocyte ghost causes stimu-lation of some of the enzymatic activity but as S.D.S. concen-tration is increased the enzymes are denatured and lose activity.

Direct measurement of other effects of S.D.S. on the ghosts is not simple because of the insolubility of membrane materials in the absence of the perturbant and also the membrane, being heterogeneous, begins to dissociate as the detergent concentration increases and disrupts hydrophobic interactions (Wallach, 1969).

Dissociation of biological membrane materials with non-ionic detergents had been previously discussed (see Section 8.4 "The use of non-ionic detergents as solubilisation agents"). The consequences of this reaction have received little attention (see Section 9.2 "Disc Electrophoresis in Polyacrylamide Gel"). Presumably the foregoing notes on general detergent action on proteins can be expected to apply because hydrophobic reaction sites appear to be of prime importance. Therefore non-ionic detergents should be reactive with membrane proteins but the contribution of a charged group to the products of the reaction is absent (Swisher, 1970). Possibly these detergents act by altering the solvation of the protein molecule and in this way may affect its properties (Dowben, Koehler and Barrieux, 1961). Such effects are likely to be much less dramatic than in the case of S.D.S., however.

10.4: Characteristics of the Detergent:Protein Complex

In view of the foregoing discussions it is reasonable to expect that, under the conditions of experiment used in the present study, detergent will be bound to the protein materials. The complex formed should be that having the maximum degree of binding which is characteristic for the particular protein because the detergents are used at a suitably high concentration. Characterisation of proteins solubilised in this manner will therefore be based on observations made on the properties of the detergent/protein complexes formed.

The final product of detergent action on protein has not

been well characterised (Tanford, 1970). In an effort to gain some knowledge of these complexes, detergent:casein complexes were prepared in the fashion that is normal to this study and were then briefly examined. This examination gives an indication only of the action of detergents on proteins and can be related only in a general way to the properties of detergent-membrane complexes. For example casein is stable and water-dispersable and thus differs in many of its properties from the membrane proteins. The information obtained is of marginal value and is presented merely to indicate the effects of detergents under the conditions of experiment.

Table 8. General Properties of Detergent:Casein Complexes

Property	Normal Casein	Casein:S.D.S. complex	Casein NID Complex
pH of precipitation	4.6	1.6 (Slight pptn. begins at pH 3.6)	Stable
Stability to Ca^{++} (0.1 ml of 1% $CaCl_2$ added to 10 ml at pH 7)	Stable	Unstable	Stable

The reduction of iso-electric point is readily explained by assuming that the S.D.S. binds hydrophobically onto the protein and this does not affect its ionic head so that it contributes by increasing the overall electronegativity of the complex. The effect of calcium could be one of reducing this electronegativity through ion binding. A similar though less extensive reduction of iso electric point occurs with bovine serum albumin (Pallansch and Briggs, 1954).

The reactions of the NID:casein complex are consistent with the view that the detergent creates an hydration envelop

in the environment of the protein molecule so that stability is maintained even when the protein is in iso-ionic condition.

The detailed nature of the S.D.S./Protein complex has been partially elucidated from a number of approaches.

a) <u>Charge of the Complex</u>

On subjecting the S.D.S. complexes of bovine serum albumin (Pallansch and Briggs, 1954) and of beta-lactoglobulin (Hill and Briggs, 1956) to free boundary electrophoresis, the mobilities of the complexes formed are found to be greater than for the native proteins. This is presumably a consequence of both the charge of the complexed S.D.S. and the native charge of the proteins acting together. In the present study, electrophoresis and DEAE chromatography of the S.D.S.:Protein complexes would differentiate them according to the charge contributed from both sources.

The fact that the S.D.S. solubilised biological membrane proteins will migrate on electrophoresis as protein:S.D.S. complexes has been recognised by a number of workers (Laver, 1964; Summers, Maizel and Darnell, 1965).

b) <u>Size of the Complex</u>

Ultra centrifugal studies of the complex of S.D.S./Beta-lactoglobulin show neither aggregation nor fragmentation of the protein (Hill and Briggs, 1956) and the hydrodynamic properties of the complex indicate that no dramatic expansion of the domain of the protein molecule accompanies denaturation by detergents (Tanford, 1968). The passage of the S.D.S. complexes of casein components through Sephadex G 200 gels also shows there is no increase in the size of these components (Cheeseman, 1968).

The size of the complex will therefore be similar to the

size of the native protein and this size would be indicated by the electrophoretic mobility of the complex through polyacrylamide gels. A good estimate of protein molecular weights can be made by observing the effects of varying the gel concentrations upon the electrophoretic mobility, while holding the charge on the protein constant (Jeffery, 1970).

A simplification of this procedure is achieved by assuming (as has been done by workers listed below) that the differences in charge between the various S.D.S.:Protein complexes will be minimal and that the complex will migrate through polyacrylamide gels as an anion with its mobility hindered only by the sieving effect of the gel structure. The mobility of the S.D.S.:Protein complex should therefore relate directly to molecular size. By studying the mobility the M.W of a protein of unknown molecular weight can be deduced (Shapiro, Vinuela, Maizel, 1967). Such and approach has proved useful in determining the approximate molecular weights of unknown proteins (Marchesi, Steers, Marchesisi and Tillack, 1970; Kennedy, 1969).

The assumption that proteins having the same charge but different sizes can be separated by the sieving effect of polyacrylamide gels has been substantiated (Hedrick and Smith, 1968) but the assumption that the S.D.S. complex with a variety of proteins will have nearly identical charge is unsubstantiated.

e) <u>The Number of S.D.S. ions bound to Protein</u>

The maximum number of S.D.S. ions that can be bound to a protein molecule can be determined and appears to be a characteristic of the protein. Some maximum molar ratios

FIGURE 1. EFFECTS OF DETERGENTS UPON CASEIN

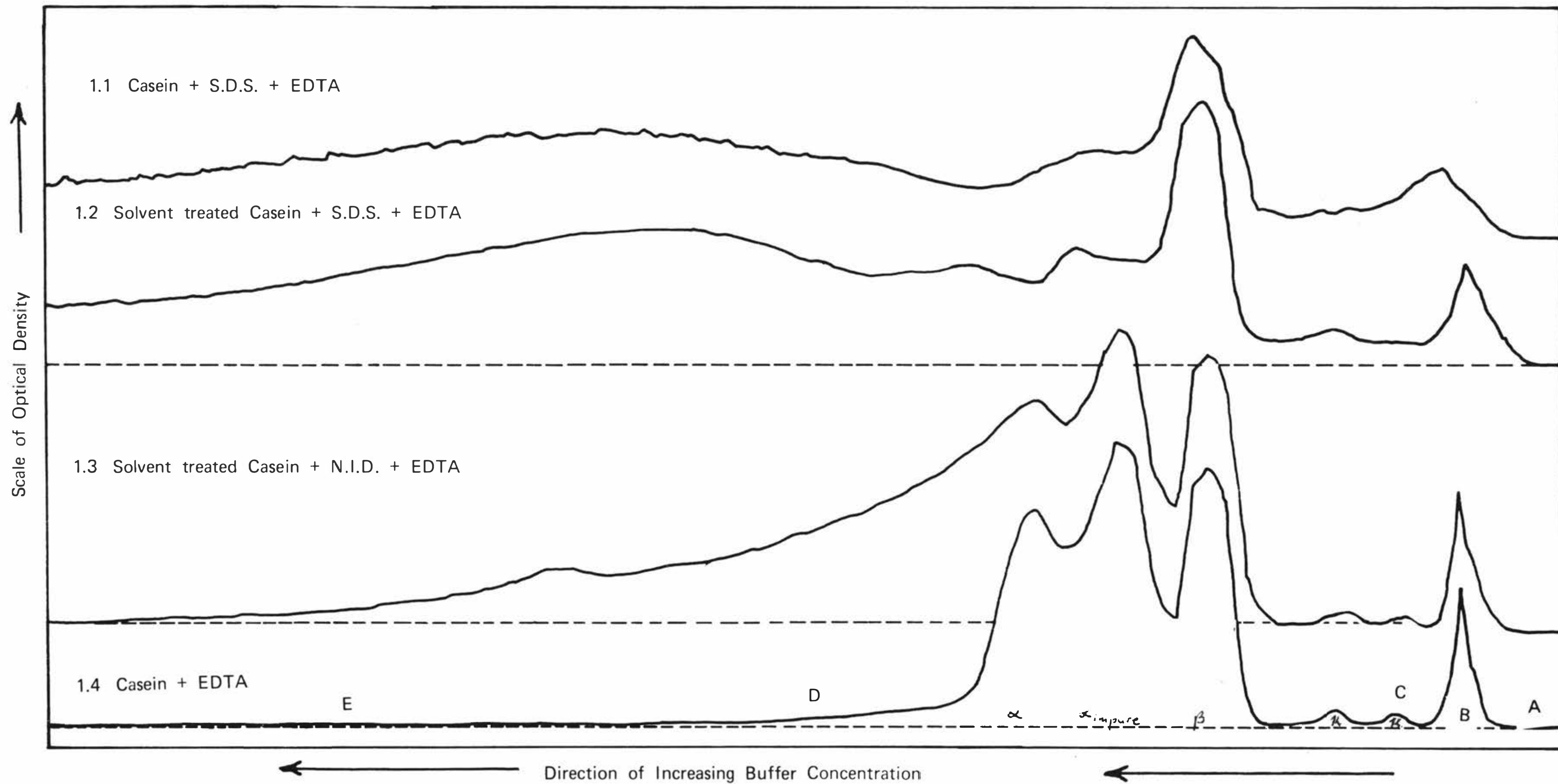


FIGURE 2. EFFECT OF DETERGENTS ON NATURAL FAT GLOBULE MEMBRANE

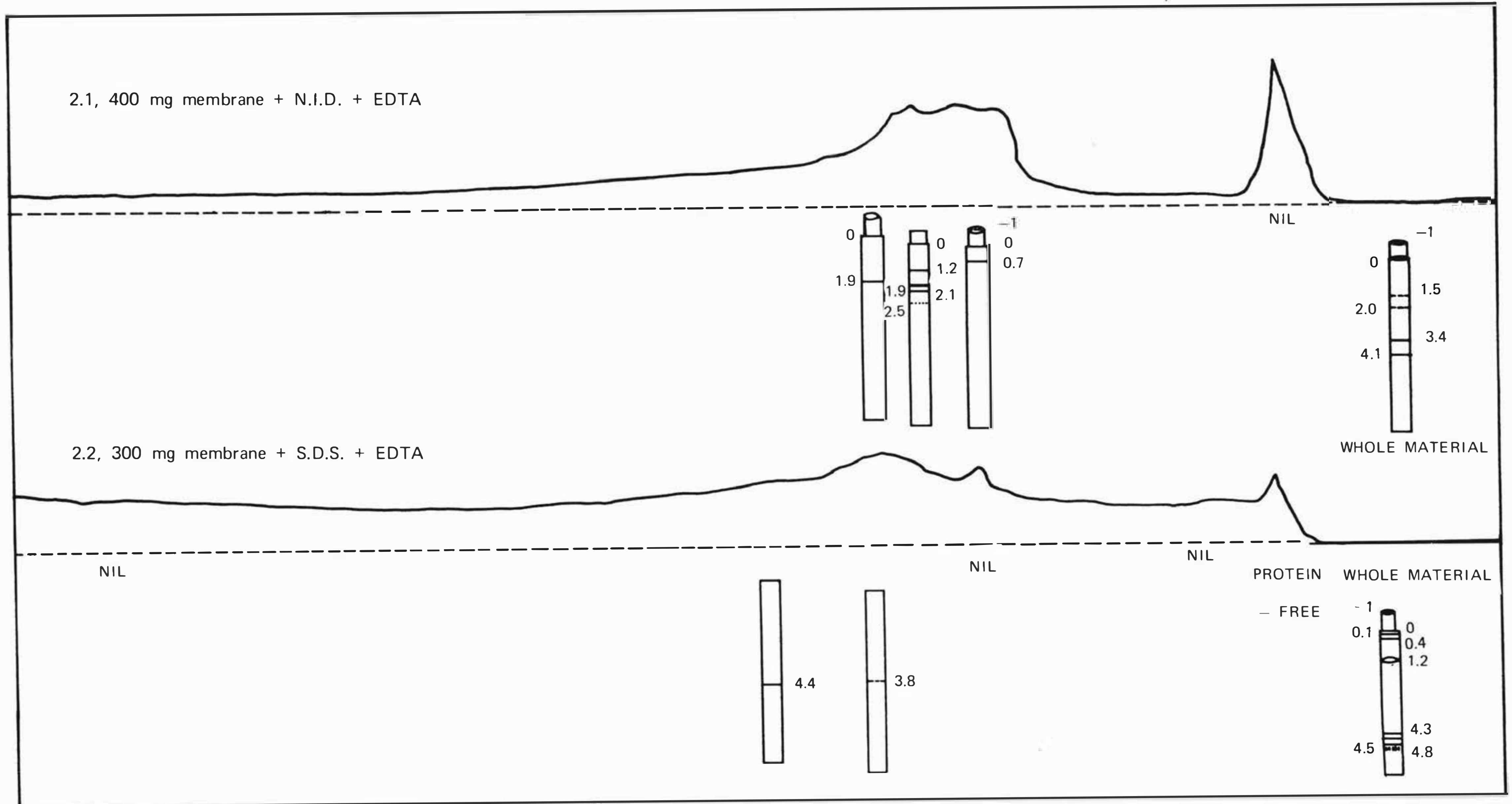


FIGURE 3. EFFECT OF DETERGENT ON THE MINOR FRACTION

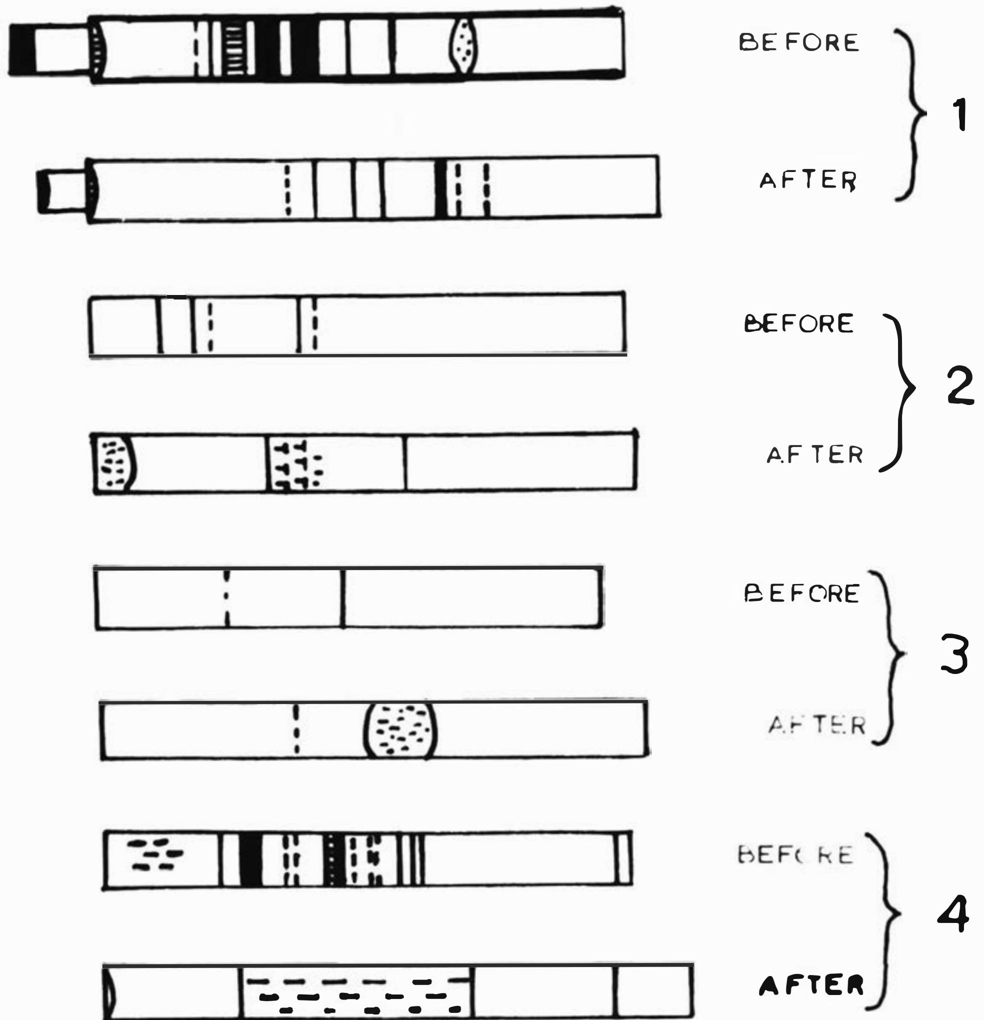
of S.D.S.:Protein are: 200 - 220 : 1 for bovine serum albumin, 50 : 1 for glycoprotein 376 of blood (Pitt-Rivers and Impiombato, 1968), 244 : 1 for β casein, 107 : 1 for Alpha$_s$ casein, 73 : 1 for the reduced K-casein (Cheeseman and Jeffcoat, 1970). These numbers are variable according to the environment in which the tests are performed which could be due to either the properties of micelle formation or to variations in the conformation of the proteins.

If it is true that the number of S.D.S. molecules bound to protein is a characteristic of a protein then it should follow that the charge of the resultant complex would vary with the number bound. This ought to influence the electrophoretic mobility of the complex and so the contention that the molecular weight of the complex can be directly inferred from its electrophoretic mobility in polyacrylamide gel is incorrectly based. The pattern of charge distribution on the protein consequently assumes particular interest for the interpretation of results in the present study. The charges exhibited by casein and membrane proteins is illustrated by the patterns of desorption of the S.D.S. complexes from DEAE columns. See Figures 1, 2 and 3.
(To assist in interpretation of the Figures - see Appendix 3 "Conventions employed in presentation of graphical results).

In every case it is immediately apparent that protein material with a wide range of charge characteristics has been desorbed from the DEAE cellulose. By comparing the patterns of casein, as dissociated by urea and mercaptoethanol (Fig. 1.4) with that of the patterns of casein dissociated by first treating with S.D.S. and then with urea and mercaptoethanol (Fig. 1.1, 1.2) the inescapable conclusion is that the protein material

has been very significantly altered and the S.D.S.:casein complex while still separable into component complexes having various charges, has a high proportion of its protein in a highly charged form. Such material does not desorb at concentrations of salt (below 0.3 Molar) that would cause the desorption of urea-dissociated casein. This is consistent with the view that S.D.S:casein complexes carry the charge of both detergent and protein and can be separated according to the charge associated with these complexes. Electrophoretic investigation of the S.D.S.:casein complexes (Fig. G) supports these conclusions. It is therefore unreasonable to infer that the electrophoretic migration is influenced only by molecular dimensions (see above (b)) and such an inference is not drawn in the present study.

Figure D. Effect of Storage of Membrane Powders at -15°



D.1 is a material extracted from the minor fraction of homogenised milk.

D.2 is a material extracted from un-homogenised milk.

D.3 is a material extracted from whey homogenate.

D.4 is a material extracted from casein homogenate.

The Influence of a Variety of Treatments upon the Properties of Membrane Materials

Before further discussion of the influence of detergent upon proteins and membrane proteins it is valuable to recognise some of the procedural factors that can alter the nature of the protein:detergent complexes and therefore influence the interpretation of results obtained. The procedure that has been developed to investigate the various materials has been carefully standardised so that comparison of results obtained from different materials can be made in the confidence that differences observed are not due to differences of technique. In the course of the investigation the following factors have been recognised as important:

a) The Effect of Storage of the Solvent-treated Membrane Powders

After extraction of fat from the freeze-dried cream powders by acetone and butanol the prepared membrane materials were stored in air-tight bottles in the dark at -15°C. Never-the less interaction of the component materials during storage was detected. Gradual insolubilisation apparently occurred and the polyacrylamide gel electrophoretic patterns of membrane powders stored for over 1 month differ from those of freshly extracted powders. This was shown by investigation of 4 different types of materials before and after storage (Fig. D).

It appears that after storage, dissociation of the materials is less complete so that sharp definition of the protein bands is not achieved. In particular, clear definition of the slow moving bands is not achieved after storage. It could be assumed that materials having large molecular size have associated so that they can no longer enter the gel. (This confirms the effect noted on loss of enzyme activity during storage). There was no evidence of any such effect of storage upon the freeze-dried creams where presumably aggregation reactions between protein molecules are inhibited through their prior association

with the fat. Similarly, dried casein does not show changes during cold storage and it is apparently a distinctive property of the homogenate that this occurs. A similar loss of solubility of fat-extracted proteo-lipids that have been stored has already been noted (see Page 72).

On the basis of these observations only materials that had been freshly extracted were employed in the investigation.

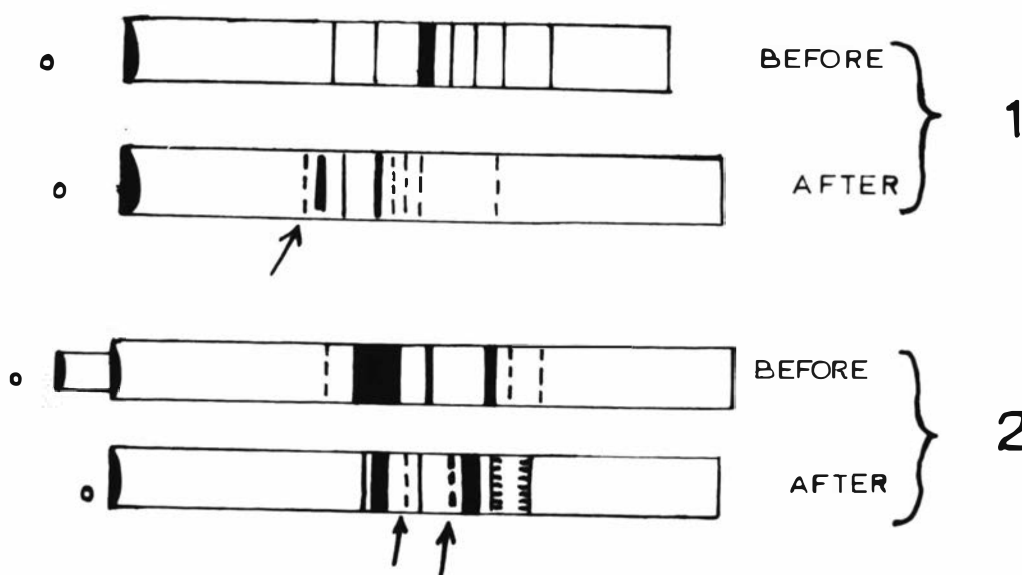
b) The Effect of Solvent Treatment upon Protein Materials

The effect of solvent treatment as employed has been shown by reference to the literature (Section 7) to be unlikely to affect the properties of the proteins. Such effects cannot be observed with membrane proteins for there is no reliable way to prepare them in a form which could act as a standard. An indication that solvent treatment does not alter the properties of pure protein is found however by observation of the nature of the S.D.S.:casein complex on DEAE-cellulose before and after treatment of the casein by solvent (see Fig. 1.1 and 1.2). The patterns are not identical but differences are small and likely to be, at least in part, due to differences in resolution achieved by the chromatographic technique. For example the roughness of the graph line at high salt concentrations is almost certainly due to difficulties with the recorder.

c) The Effect of Holding Solubilised Samples Overnight in Urea Solution at 5°

Membrane materials solubilised by non-ionic detergent, urea and buffer (but not mercaptoethanol) show changes in electrophoretic pattern after storage at 5° and in the dark.

Figure E. Effect of holding Samples, in Urea, Overnight at 5°



Both patterns represent material extracted from the minor fraction of homogenised milks. In pattern E.1 a new band has appeared. Some reduction in mobility of other bands is observable although this may be attributable to inter-gel variability because, necessarily, the same sample was run on succeeding days. In pattern E.2 two new bands have appeared after storage and there is a loss of definition of the two fastest moving bands.

Such observations can be explained in terms of the carbamylation of lysine groups by impurities in urea as has been observed with other protein systems (see Section 8.2). Alternatively it is possible that the non-ionic detergent is slow in reacting with some membrane components and has caused their solubilisation or alteration after overnight storage.

For these reasons it is important to use the samples within a few hours of preparation. This was particularly true for the electrophoretic observations, the chromatographic technique being less sensitive to this source of error.

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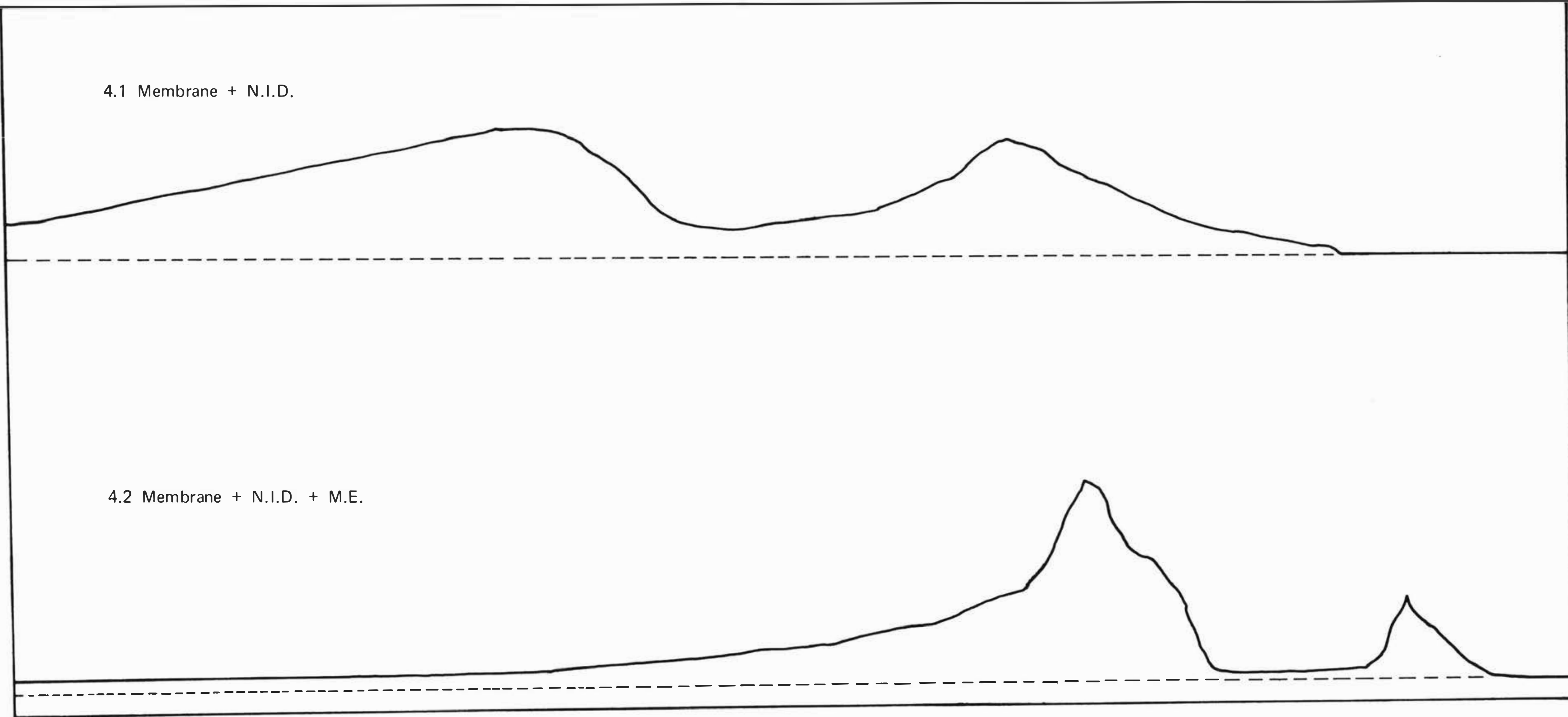
FIGURE 4. EFFECT OF 2–MERCAPTOETHANOL ON MEMBRANE (MINOR FRACTION)



d) <u>The Effect of the Presence of 2-mercaptoethanol</u>

The presence of disulphide bonds in the protein materials is to be expected from previously published information (see Section 8). The value of reduction of these bonds is illustrated by the DEAE-cellulose patterns of a NID-solubilised membrane material from the minor fraction of homogenised milk. In the absence of 2-mercaptoethanol (Fig. 4.1) this material has separated as two large peaks. After cleavage of the disulphide bonds (Fig. 4.2) the peak containing low-charged material grows, presumably at the expense of material in the highly charged peak. Conceivably large molecules have dissociated leading to smaller components having a fraction of the number of charges upon them.

The leading peak on the pattern of protein containing 2-mercaptoethanol (Fig. 4.2) appears at relatively low salt concentrations and is present in the patterns of all the protein materials tested. The material that elutes from the column at this point has been collected and investigated by polyacrylamide gel electrophoresis on a number of occasions (e.g. Fig. 2) with negative results. It does however commonly show a fairly typical U.V. absorption pattern for proteins, though this is not always so. It has been considered to be predominantly an artifact peak when investigated during studies with casein (Sanderson, Pers.Comm.) and these results tend to confirm this point. That the U.V. scan patterns do indicate proteins probably means that there is a small concentration of protein which desorbs at this point and raises the base-line of the graph but which is insufficient to give a recognisable electrophoretic band. This is not always true, for protein bands were occasionally detectable (Figs. 3.1, 13.2). Also the desorption of protein prior to this point in Fig. 5.2 probably indicates a similar situation.

e) <u>The Effect of Including Detergent in the Elution Buffer During Chromatography</u>

As dialysis is effective in dissociating the detergent: protein complexes it is possible that when passing buffer over the complexes while they are bound to the DEAE-cellulose beads that they might begin to dissociate during the period of chromatography. Such partial dissociation of a S.D.S.: Protein complex would cause loss of charge of the complex until it desorbed for this reason. Concomitantly desorption will also be occurring because of the gradual increase in sodium chloride concentration. Such reasoning could well explain the material which appears as a long low profile in the high salt concentration end of the patterns of the S.D.S.:Protein complex (Fig. 1.1, 1.2). Similar long low profiles appear with a number of the materials complexed with S.D.S. Such dissociation could be only partial because membrane materials would be unstable in the absence of detergent and would precipitate. Furthermore excess detergent is present in the sample and would bind onto the DEAE-cellulose in the vicinity of bound protein.

This would mean that the environment of the bound complex would not be devoid of detergent so dissociation of the complex should not begin immediately.

To investigate this point and so perhaps to improve the separation an attempt was made to elute the S.D.S.:Casein complex in the presence of S.D.S. The normal procedure was followed except that 1% S.D.S. was added to the elution buffer. The presence of S.D.S. definitely altered the results (see Fig. 5). It appears that in the presence of S.D.S. desorption has occurred at lower salt concentrations than when it is absent. This does not conform to the above expectations for the presence of S.D.S. ought to maintain the S.D.S.:Protein complexes so that they retain their high charge and so desorb

at a later stage. That the opposite effect has occurred can be explained by assuming that the S.D.S. anion has adsorbed in competition with the protein complex and so has contributed to its rapid elution.

At this point either technique is suitable for separation of the protein components provided the information obtained is correctly interpreted. The preferred technique was decided by practical difficulties. The level of 1% for S.D.S. in the buffer exceeds the critical micelle concentration for this detergent at room temperature and it is present in micellar form. Addition of S.D.S. to the buffer compartment containing the 2 Molar sodium chloride causes an increase in micelle size to such an extent that the micelles become readily visible. (This increase in micellar size with increasing electrolyte concentration is a physical property of micelles. (Elworthy, Florence and MacFarlane, 1968). As the sodium chloride level builds up in the eluting buffer some micelles adsorb onto the top of the DEAE column to form an insoluble layer of detergent which progressively blocks the further flow of buffer and stops the separation well before it is completed.

Addition of non-ionic detergent to the buffer while eluting the NID solubilised material also proved impractical because the mixture absorbs ultra-violet light strongly and the pattern of the protein fractions is obscured and interpretation of results becomes arguable. For the purpose of the investigation, therefore, elution without detergent in the elution buffer is used. Any effect of dissociation of the detergent:protein complex should be the same for comparable peaks in different materials so comparison of the patterns obtained from different protein sources should still be valid. That patterns of materials solubilised by non-ionic detergent do not show the long low profiles at high salt concentration which appear with S.D.S.:Protein complexes supports the

interpretation that loss of charge due to dissociation of the S.D.S. from the complex does occur.

In the elution patterns of the NID:Protein complexes the elution of excess NID originating from the sample may contribute at some point to the shape of the patterns. No such effect common to all experiments is, however, detectable. The pattern obtained with the NID:natural membrane provides critical information in this regard because there is very little protein actually present and since the pattern does not show an unexplained peak carrying no protein, the effect cannot be significant (Fig. 2.1).

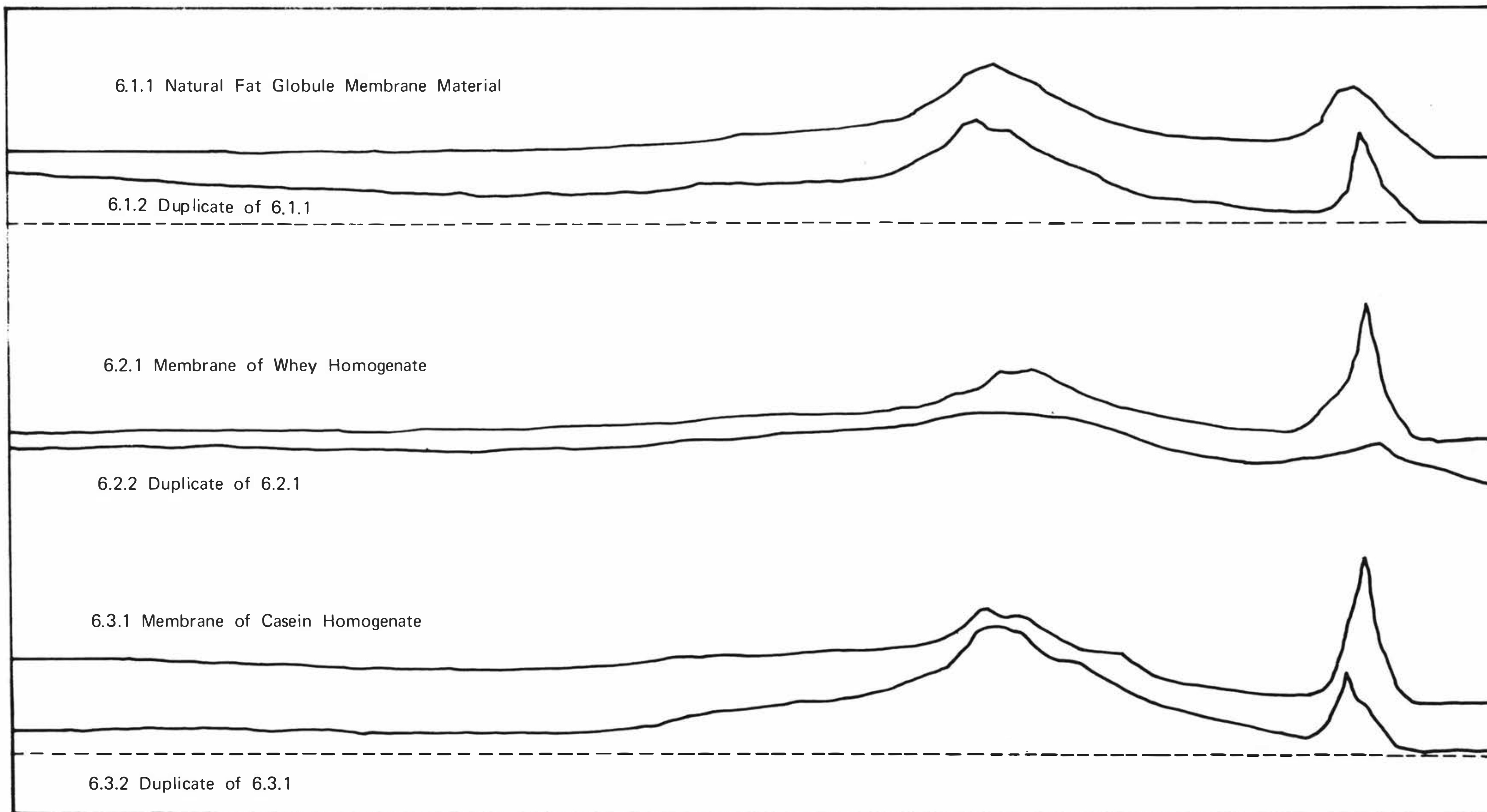
f) The Possibility of Further Variables that are Unidentified

The validity of comparing the chromatographs and electrophoretic patterns obtained from differing materials depends upon the extent of replication of results that is achievable. Not only the effects of variables in technique but also variability of operations inherent in the entire system of the investigation need to be evaluated. This has been performed in a number of ways and can be demonstrated most usefully by first, observing the duplication of results of chromatography of a S.D.S.:Protein complex, and secondly, by observing the replication of results of NID solubilisation of three natural fat globule membranes as shown by polyacrylamide gel electrophoresis. Additionally the very fact of demonstrating the effects of the variables shown above illustrates the sensitivity of the techniques used.

i) Duplication of Results of DEAE-cellulose Chromatography of the S.D.S. complexes of Various Materials (Figure 6)

The patterns presented represent three membrane materials that have been solubilised and chromatographed

FIGURE 6. DUPLICATION OF CHROMATOGRAPHY PATTERNS OF S.D.S. COMPLEXES



in duplicate from the same solvent extracted powders. Duplications were made within a fortnight of one another. The identity of the materials used was:

Fig. 6.1 A natural fat globule membrane material

Fig. 6.2 Membrane material from whey homogenate

Fig. 6.3 Membrane material from casein homogenate

Fig. 1.1, 1.2 Pg 106 Casein.

Two principal observations can be made. First the leading peak (which is probably an artifact of 2-mercapto-ethanol) varies in sharpness and height and when it is least distinct (presumably because of a low level of 2-mercaptoethanol) (Fig. 6.2) the definition of the protein peaks is not very satisfactory. This tends to support the view that excess mercaptoethanol is essential to clear separation.

Secondly, the separation of protein components is, in general terms, the same within every pair and differences appear principally in the definition of peaks. Clearly, however, it is not possible to place emphasis on minor peaks when comparing the DEAE chromatograms of different materials after S.D.S. solubilisation. The significance of these patterns rests on two criteria; differences in major peaks and differences in the electrophoretic characteristics of fractions collected from the column.

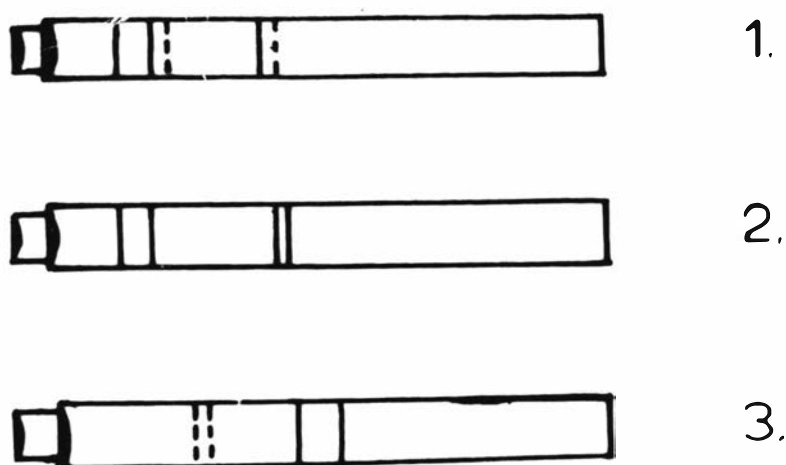
Definition of the S.D.S. complexes of entire membrane materials by electrophoresis was not satisfactory and consequently such results are not presented. The characterisation of these complexes therefore depends upon chromatography. Nevertheless the electrophoresis of membrane components after fractionation and subsequent preparation gave fair definition and was repeatable though

there were a high proportion of "Nil" results.

ii) <u>The Replication of Results with Natural Fat Globule Membranes, solubilised by Non-ionic Detergent and Characterised by Polyacrylamide Gel Electrophoresis</u> (Figure F)

The most sensitive check available on the reliability of the results of the investigation is demonstrated as follows (Fig. F):

Figure F. Characterisation of Natural Membrane, Solubilised by N.I.D. in polyacrylamide gel electrophoresis



Nautral membrane materials were preapred from three unhomogenised milks, solubilised and characterised. Four comparable bands of protein appear in each case though in one case (F.1) a fifth faint band has been distinguished. The migration distance of the four principal bands in Fig. F.1 and F.2 are identical though a higher rate of migration has occurred in Fig. F.3.

Such definition of the components of natural fat globule membrane has not been previously reported. Obviously total solubilisation has not been achieved for

material has been held up at the points of entry to each gel. This infers that the natural fat globule membrane protein is quite heterogeneous being similar to many natural membranes in this respect. The particular relevance of these results to the present study is that the entire system of membrane isolation, preparation and analysis has been checked by the most sensitive technique and found to give good repeatability of results. Comparability of membrane materials is therefore assured though some reservations must be retained about the distance of migration of the components and about the significance of faint bands. For these reasons the electrophoresis of NID solubilised membrane materials was invariably carried out in duplicate at least.

Section 12

The Effects of Detergents upon Protein Materials

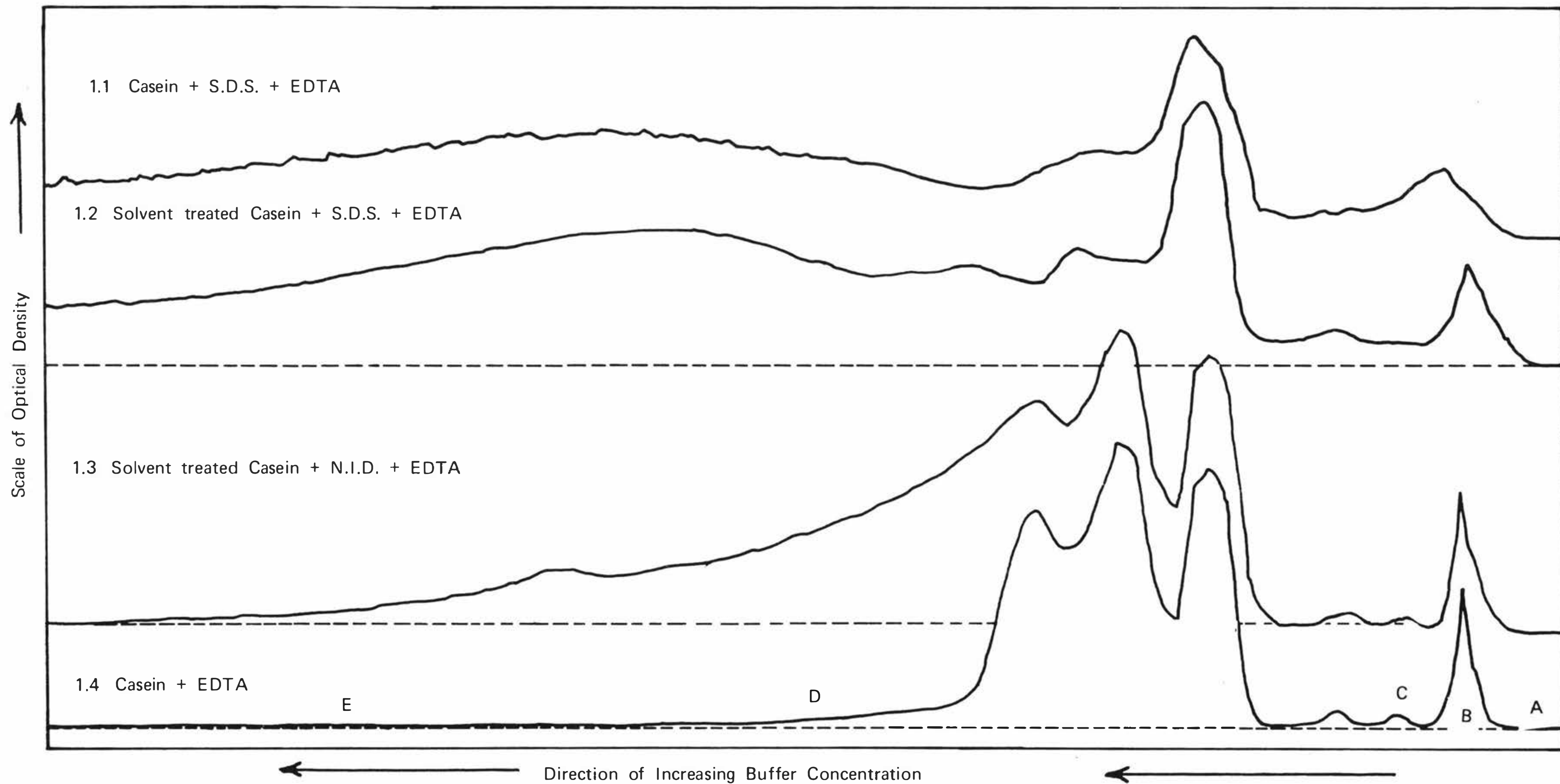
It has been established that, (a) the use of S.D.S. to solubilise membranes causes dissociation and creates S.D.S.: Protein complexes carrying a variety of charges and which can be separated by chromatography on DEAE cellulose. A technique to distinguish these complexes has been developed and standardised following recognition of the interplay of the various factors affecting the properties of the membrane materials, (b) a technique for the solubilisation of membrane materials by a non-ionic detergent mixture has been developed and the solubilised materials can be characterised by electrophoresis and by DEAE chromatography.

Study can now be made of the effects of detergents as used in the present study upon a variety of membrane materials and an interpretation of the results can be developed.

12.1. The effect of detergents upon native milk proteins

The aim of this aspect of the study is to recognise some of the properties of detergent complexes and to establish any

FIGURE 1.   EFFECTS OF DETERGENTS UPON CASEIN



similarity of behaviour of the membrane materials. The effect of S.D.S. upon casein is illustrated by DEAE chromatography (Fig. 1.1 and 1.2 compared to 1.4) and by electrophoresis (Fig. G).

Figure G. Effect of S.D.S. on Casein

1.) CASEIN

2.) SDS- TREATED CASEIN

It is shown that S.D.S. causes major alteration to the nature of the casein. A higher charge has been imparted to much of the protein and this is consistent with the assumption that various components combine with different amounts of S.D.S. and the complexes are subsequently distinguished on the basis of their different charges. This is supported by evidence that the casein components bind S.D.S. to different extents (Cheeseman and Jeffcoat, 1970). The DEAE patterns appear superficially to show that beta-casein and kappa-casein peaks have not been altered by the presence of S.D.S. but this is shown by the electrophoretic patterns (Fig. G) to be coincidental because these two components show higher than normal mobilities and it seems unlikely that they could appear at the same points on the chromatogram as they would when not complexed with S.D.S.. It is notable too that electrophoresis in the presence of S.D.S. under the conditions used does not produce sharp protein bands which may indicate

that S.D.S.:Protein complexes interact with one another in some way during electrophoresis. Alternatively, it may merely indicate that the electrophoretic conditions used were unsuited to separation of these complexes.

The presence in the chromatograms of protein having relatively low charge in the areas normally representing kappa-casein is perhaps surprising because all complexes should be more highly charged than native materials and because very little low charged material is represented in the electrophoretic results. The effect of S.D.S. competition for binding sites on the DEAE-cellulose has been proposed and may explain such early desorption of some components.

The long low profile of highly charged material on the chromatograms has already been commented upon. It may indicate highly charged S.D.S. complexes that progressively desorb under the joint influence of increasing sodium chloride concentration and decreasing S.D.S. levels as S.D.S. is lost from the complex to the elution buffer thereby causing a progressive loss of charge of the complex.

The conclusion to be drawn from these observations is that S.D.S. causes a major alteration to natural protein and establishes that the resultant S.D.S.:Protein complexes are entirely different from the original material. The duplication of results which is achievable does support the assumption that the ability of a protein to bind S.D.S. is a characteristic of that protein and can be measured by charge.

The presence of the non-ionic detergent mixture in a casein suspension has caused only a minor change in the nature of the casein as detectable by chromatography (Fig 1.3). The alpha-casein peak appears to have been altered and shows a

proportion of material having higher than normal charge (compare Fig. 1.4). This is unlikely. If non-ionic detergents do associate hydrophobically with protein the more hydrophobic beta-casein is more likely to be the point of association and also a loss of charge might be expected because the affect would be an increase of solvation. Possibly the effect is an artifact for, as previously noted, the NID in the buffer absorbs light at 280 nM strongly and evidence has been obtained that the NID used did elute at this point.

The effect of NID on whey protein has been investigated by polyacrylamide gel electrophoresis (Fig. H).

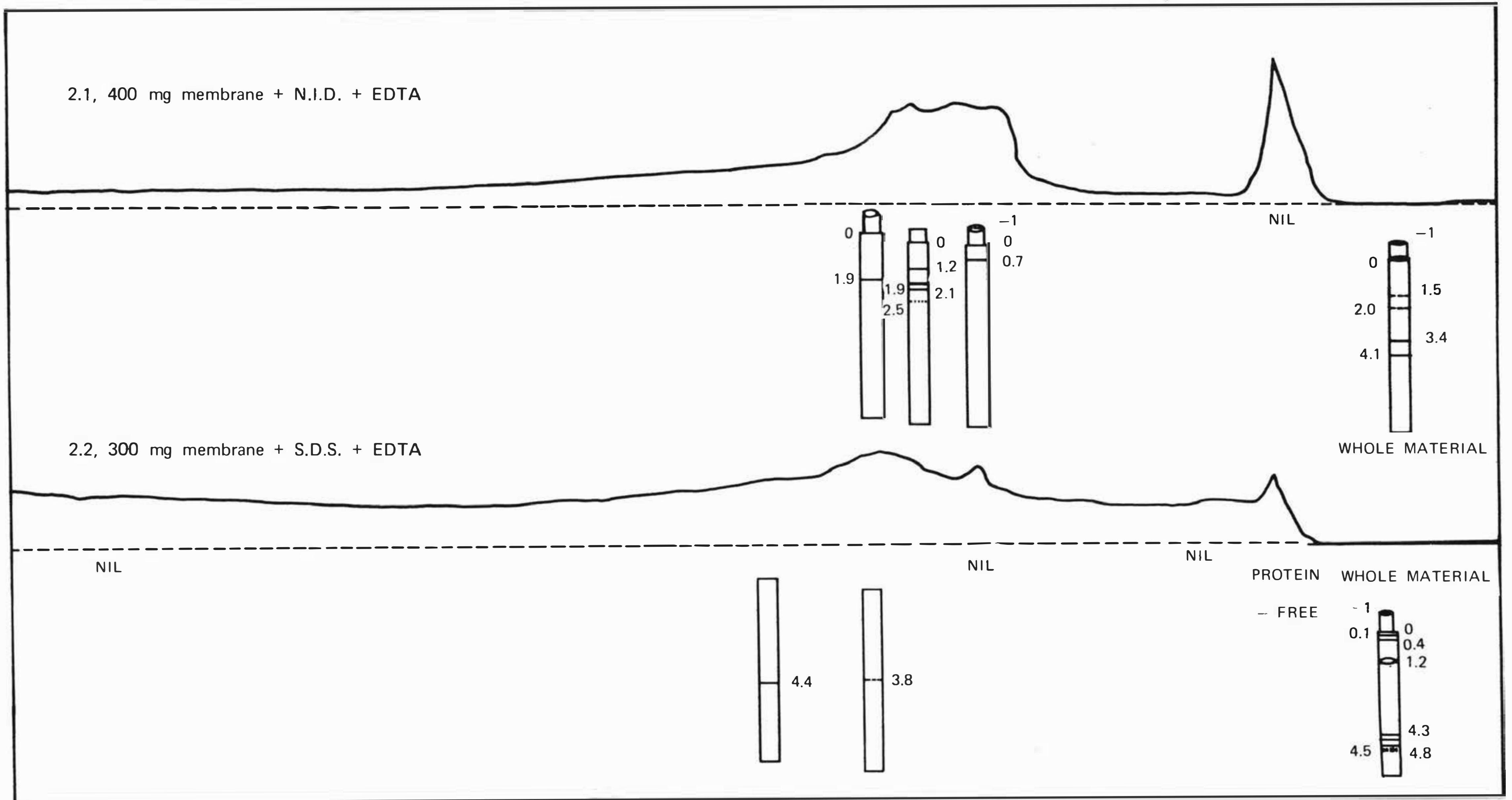
Figure H. Effect of N.I.D. on Whey Powder

1. Whey Powder

2. Whey Powder with N.I.D.

The results presented accord with observations in the literature that the non-ionic detergents do not have a major effect on the properties of native proteins as observed by chromatography or electrophoresis. Some caution may be indicated in interpreting the level of chromatography peaks that desorb at about 0.4 Molar sodium chloride. While error at this point may be important in absolute terms, it will provide a constant error contributing to such peaks and will be acceptable for comparative purposes. Nevertheless justification, by electrophoresis of fractions at this point, is important.

FIGURE 2. EFFECT OF DETERGENTS ON NATURAL FAT GLOBULE MEMBRANE



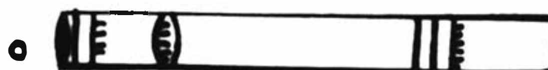
12.2. The effect of detergent upon natural fat globule membranes (Figs. 2 and I)

The aim of this study is to provide recognition of the contribution that the patterns of natural globule membrane patterns may make to the patterns of the membrane of homogenised milk and only secondarily is it a study of the nature of natural fat globule membrane materials.

Electrophoresis of a whole membrane material extracted from one unhomogenised milk and solubilised in the two detergents leads to very different results (Fig. I).

Figure I. Effect of Detergent on Natural Membrane

1. In Presence of S.D.S.



2. In Presence of N.I.D.

Electrophoresis of the S.D.S. complexes is difficult and sharp definition of bands has not been achieved. Nevertheless rather more component bands appear after S.D.S. solubilisation than after NID solubilisation. This accords with the poor gross solubility in the NID that was chosen because of this property.

The significance of this results is tempered by the observation that chromatography (Fig. 2.1) separates three peaks from the NID solubilised membrane and that on electrophoresis these heterogeneous peaks yield components having

different mobilities to those detected in the material prior to chromatography.

NOTE: Components may be identified as follows:

Component No.	Peak No.	Migration Distance
1	1	-1
2	1	0.7 cm
3	2	1.2
4	{ 2 { 3	1.9 1.9
5	2	2.1
6	2	2.5

It may be that chromatography assists separation in that components that are able to associate in the whole material and possibly cannot enter the gels, are separated into fractions by the adsorption:desorption conditions so that they cannot thereafter reassociate thereby behaving as individuals having their own characteristics on polyacrylamide gel electrophoresis. Alternatively the dialysis and concentration prior to electrophoresis of the fractions may alter their electrophoresis properties.

The relationship between the electrophoretic patterns of the whole S.D.S.-protein material and those of the fractionated materials has also been disturbed (Fig. 2.2). Only two components of the fractions from chromatography of the S.D.S. complexes are detectable by electrophoresis. The dialysis procedure is very likely to have created this effect for as S.D.S. is dialysed free of the complex the charge and consequently the migration characteristics would alter. Furthermore, it is possible that reassociation of proteins in the presence of the relatively lower level of S.D.S. could

prevent entry of the complex into the gels and this would lead to a "Nil" result. Electrophoresis of fractions is therefore useful to detect heterogenity of fractions and to prove the presence of protein, but is open to confusion if migration rates alone are considered characteristic of a component protein.

The chromatogram of the NID-solubilised membrane (Fig. 2.1) shows a low recovery of protein from the 400 mgm of membrane material that was initially used. This observation occurs because the HLB value chosen for the NID was one at which the membrane created by homogenisation was effectively solubilised but the natural membrane had very low solubility. This effectively fractionated the two membrane types. The S.D.S. solubilised membrane has also yielded low levels of protein but in this case the peaks are less sharp and the total protein eluted is greater.

In both cases, but most noticeably in the chromatogram of the S.D.S. solubilised membrane, material that absorbs light at 280 nM continues to desorb from the column to very high sodium chloride levels. Extending the assumptions previously drawn it appears that highly charged S.D.S.:Protein moieties of the natural fat globule membrane are formed from protein moieties that are highly hydrophobic in nature and which readily associate with other hydrophobic materials. This would explain the change of properties that occur on storage as being due to the gradual build up of hydrophobic linkages. On exposure to detergents hydrophobic reaction could readily occur which accords with the effectiveness of detergents as dispersants of these materials. This is true of non-ionic detergents which are not normally noted for their reaction with proteins (though they do react with lipo-proteins) and the reaction is most satisfactory for detergents having hydrophobic

properties. If this is correct the more hydrophobic molecules are likely to associate with a high number of S.D.S. molecules giving a proportion of complexes with high charge. On chromatography these dissociate slowly under the effect of dilution by the eluting buffer and eventually desorb as the sodium chloride level increases. This effect appears on the chromatogram as a long slope upwards away from the baseline and probably not all material has desorbed even at the 1.2 M sodium chloride concentration (Note: The upward slope is not a characteristic of chromatography due to gradual alteration of baseline, because this effect is not apparent in the patterns for casein in Fig. 1.3 and 1.4). In the presence of NID the amount of highly charged material is less and the slope of the line is slightly downwards. Possibly there is no protein present for the reaction of NID with casein leads to some doubt of the validity of the graph in this region because NID also absorbs at 280 mM and may be eluting in this region.

The conclusions drawn are that the two detergents react differently with natural fat globule membrane and each will provide information that is complementary to the other. The electrophoretic characteristics of whole membrane materials and those of dialysed fractions after chromatography did not yield comparable information. After complexing with S.D.S. a proportion of the material exists in highly charged form.

The partially solubilised natural fat globule membrane protein has been fractionated and shown to contain a number of protein moieties, five of which have been detected. This indicates greater heterogenity of the protein moieties than has previously been established. (Five lipo-proteins have been centrifugally separated from the natural membrane (Chien and Richardson, 1967) but the above comment refers to only the small proportion that is soluble in the NID used).

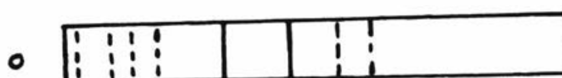
12.3. The Effect of Detergent upon the Membrane Material of Homogenised Milk (Minor Fraction)

The aim of this study is to recognise the influence of detergents upon the properties of the protein materials that have been isolated from the smallest globules of homogenised milk. The conditions of solubilisation, electrophoresis and chromatography have been chosen to provide clear information about these materials. As stated earlier characterisation of the native protein and natural fat globule membrane under similar conditions has proven to be adequate but not ideal.

Electrophoresis of a minor fraction membrane in the two detergents was successful (Fig. J).

Figure J. Effect of Detergent on Membrane of Homogenised Milk (Minor Globule Fraction)

1. In Presence of S.D.S.

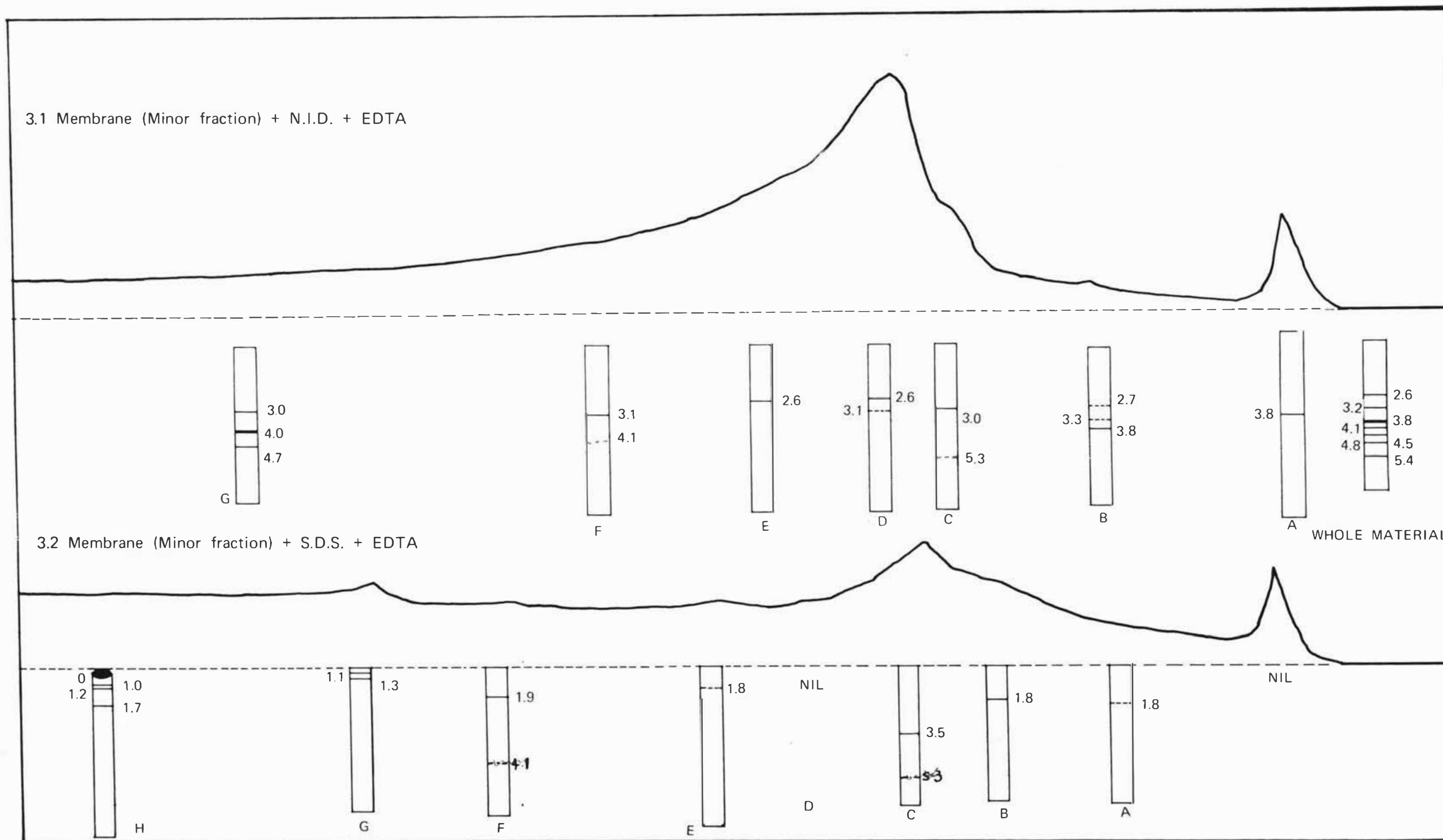


2. In Presence of N.I.D.

Each detergent has acted differently but both have demonstrated 8 components. Particularly clear bands have been produced but once again the significance of these results must be tempered by the results prepared from chromatographic fractionation (Figure 3).

The chromatography and fractionation of the NID solubilised membrane (Fig. 3.1, which is not of the same origin as Fig. I) has been particularly successful. Even the lowest peaks

FIGURE 3. EFFECT OF DETERGENT ON THE MINOR FRACTION



contain protein that can be detected electrophoretically. The chromatographic peaks are heterogeneous but the fractionation has led to the identification of a number of components that were not previously apparent. Also the migration distances of fractions and of the components of the whole material are comparable which possibly distinguishes this material from the natural membrane materials. Probably 11 components have been detected as follows:

Component	Fraction	Mobility
1	A, B	3.8
2	B	2.7
3	B	3.3
4	C	3.0
	D	3.1
5	C	5.3
6	D	2.6
	E	2.6
7	F	3.1
8	F	4.1
9	G	3.0
10	G	4.0
11	G	4.7

Materials that desorb at widely different points on the chromatogram have very different charges and therefore, if they have the same electrophoretic mobility as one another, they must have different size, and so they have been identified as different components. For example the component No. 4 appears in two fraction, C and D which are close together but it is absent from fraction E. On the other hand the components 7 and 9 have similar mobilities but widely differing charges so have been identified as different materials. These three components are presumably masked in the whole material by collecting at the single band with mobility of 3.2.

In this pattern there is no doubt that protein is contained in the long low profile of the materials desorbing at high sodium chloride concentrations. Three components are detectable at Fraction G and probably more exist at points beyond the terminating point. Even more may well have desorbed at higher sodium chloride levels and the 11 components identified are perhaps only a proportion of those that are soluble. The collection of a greater number of fractions is open to difficulties of interpretation as, for example, it is not clear whether component 6 with the same mobility at two nearby fractions is in fact two components or one.

The patterns of the S.D.S. solubilised material (Fig. 3.2) is again very different to that of the NID solubilised material. The technique is less satisfactory for the chromatographic peaks are less sharp, electrophoretic patterns of the whole material showed extensive blurring of bands and cannot be presented, bands of the components in the fractions are faint, (probably because S.D.S. interferes with the amide black staining reaction), and mobilities of the bands are less reliable because of the likely effects of dialysis (as previously explained). Despite the reservations it might be possible to recognise 11 components as follows:

Component No.	Fraction	Mobility
1	A	1.8
	B	1.8
2	C	3.5
3	D	Nil
4	E	1.8
5	F	1.9
6	G	1.1
7	G	1.3
8	H	0
9	H	1.0
10	H	1.2
11	H	1.7

This is the same number as detected by NID solubilisation which is interesting but perhaps fortuitous. The highly charged components have relatively low mobilities so are apparently large. The cautions expressed about interpretation of results for the NID solubilised materials presumably still apply and some other points have become apparent.

Component 3 is probably a real component. The chromatogram does not show a dip, and the U.V. scan indicates protein, so the "Nil" result may simply mean that the protein, or proteins, was too large to enter the gel. This has clearly occurred with component 8, where material is observable at the point of entry. "Nil" results are fairly common with the S.D.S. complexes and it may be that dialysis has resulted in a loss of stability and/or subsequent aggregation.

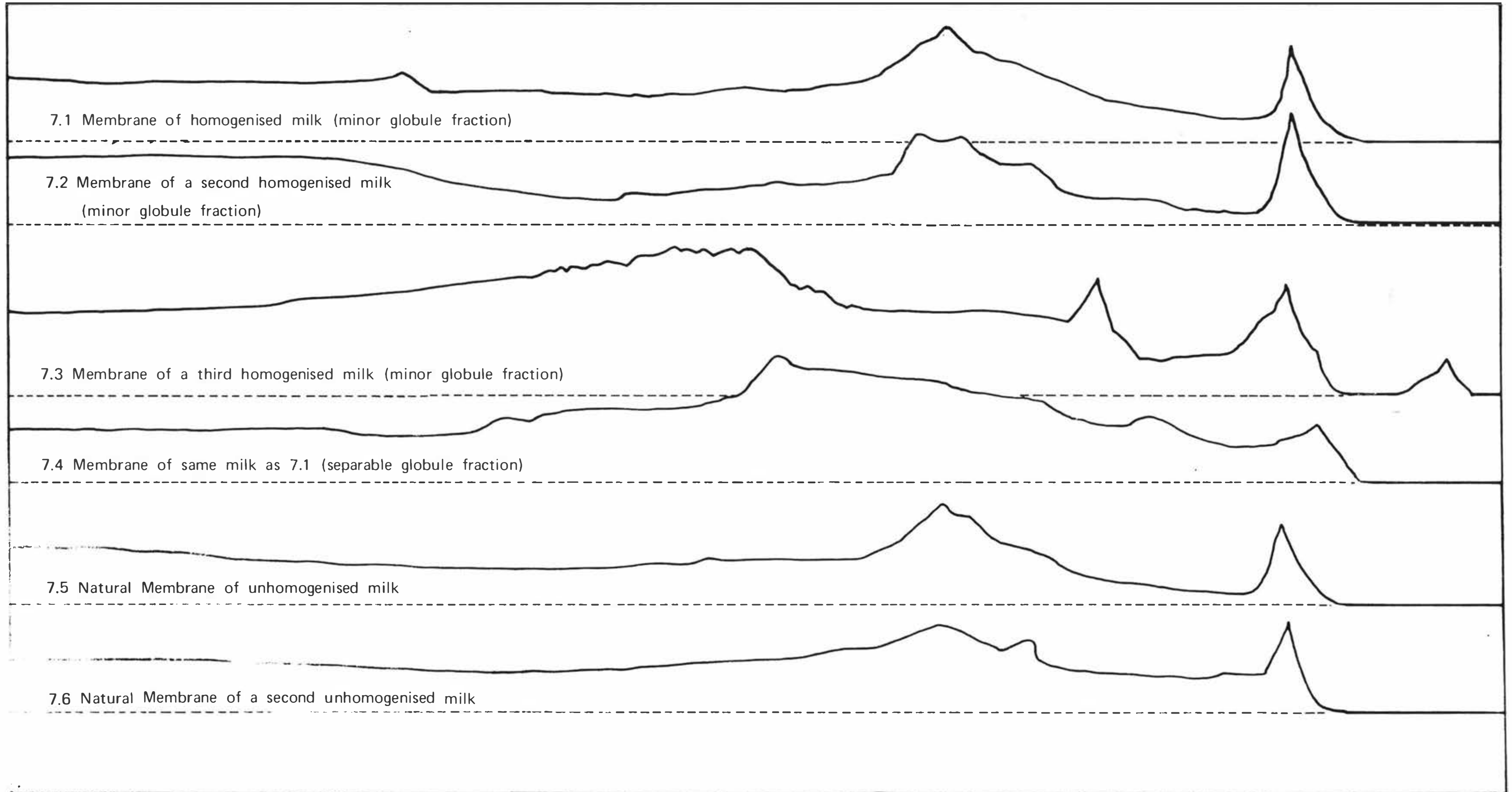
The components of fraction G and H are clear and have separated distinctly. This has not been observed for other materials so it is useful to speculate upon the nature of the chromatography process. If a hypothetical molecule A formed a complex with 200 molecules of S.D.S. ("A:200") it might well appear in fraction H with an electrophoretic mobility of 1.7. Possibly the complex "A:150" also occurs and it would desorb at lower sodium chloride concentrations and have lower mobility; at say 1.1 in fraction G. That is to say that components 6 and 11 might contain the same parent protein. This will only occur if maximum binding of S.D.S. has not been achieved but there is no reason to suppose that this is so.

Alternatively, working against this possibility is the proposal that as the eluting buffer passes over the adsorbed complex dissociation progresses gradually with loss of S.D.S. The hypothetical component "A:200" having the characteristics of component 11 could not then possibly dissociate to become

the complex "A:150" until some point after fraction H. This mechanism therefore acts to speed desorption of complexes having high S.D.S.:Protein ratios and acts in concert with the increasing level of sodium chloride. On the other hand, the formation of S.D.S. complexes with various S.D.S. levels on the same protein component is conceivable but less likely.

The assumption is made that the S.D.S.:Protein complex acts to create a high charge and this will delay elution of the complex. While this is probably true it can be seen that the charge contributed by the protein itself is very significant, for three components with high charge and high electrophoretic mobility appear among the MID fractions. Further comment will be made on this point. It is also proposed that the more hydrophobic protein materials will bind a greater number of S.D.S. molecules and so exhibit a greater charge. Late desorption on the chromatograms would then indicate greater hydrophobicity. If this is so the materials of the homogenised membrane exhibit a wide range of hydrophobicity though the charge contribution of the native protein material must obscure the correctness of this interpretation.

FIGURE 7. CHARACTERISATION OF VARIOUS MEMBRANE TYPES BY THEIR S.D.S. COMPLEXES



The Characteristics of the Materials Adsorbed onto the Fat/Plasma Interface due to the Homogenisation Process

Characterisation of the "membrane" that is created by the homogenisation process has been performed by three distinct techniques. These are:

1) Chromatography on DEAE-cellulose of the S.D.S.:Protein complexes of membrane materials
2) Chromatography on DEAE-cellulose of NID solubilised membrane materials
3) Electrophoresis in polyacrylamide gel of the NID solubilised membrane materials.

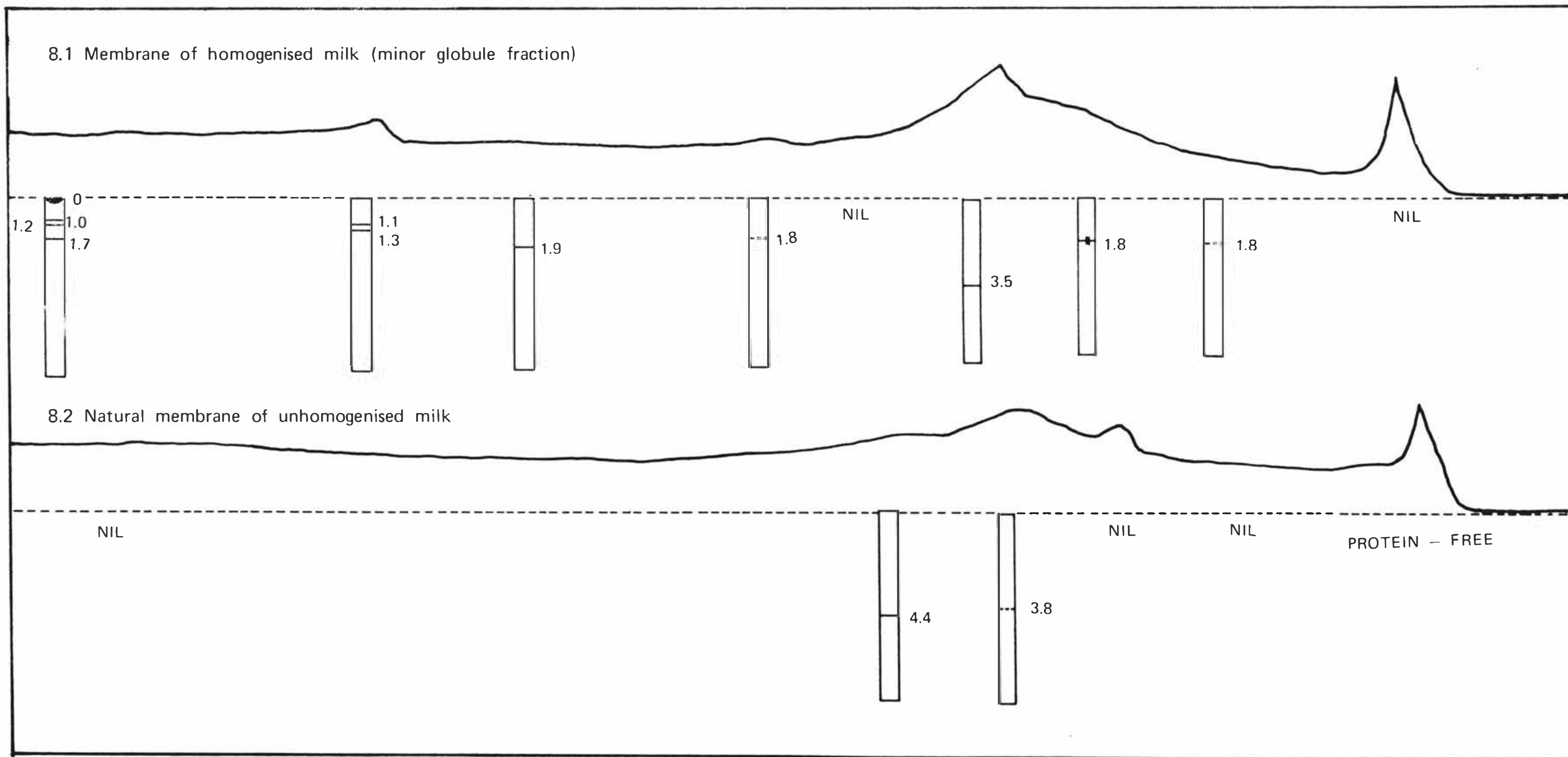
The results of these techniques have been compared to the chromatographic and electrophoretic characteristics of the membranes derived from homogenates of butterfat with casein and with whey powder. Additionally, the solubilisation properties of the homogenised "membrane" have already been reported.

13. 1) Chromatography on DEAE-cellulose of the S.D.S.:Protein Complexes of Membrane Materials - Figures 7 and 8.

The origin of materials represented by the chromatograms are:

Figure 7.1, 8.1	membrane material from the minor fraction of a milk
Figure 7.4	membrane material from the separable fraction of the same milk
Figure 7.2	membrane material from the minor fraction of a second milk
Figure 7.3	membrane material from the minor fraction of a third milk
Figure 7.5	membrane material from an unhomogenised milk
Figure 7.6, 8.2	membrane material from a second unhomogenised milk.

FIGURE 8. SIGNIFICANCE OF DEAE CHROMATOGRAPHIC PATTERNS OF S.D.S. COMPLEXES



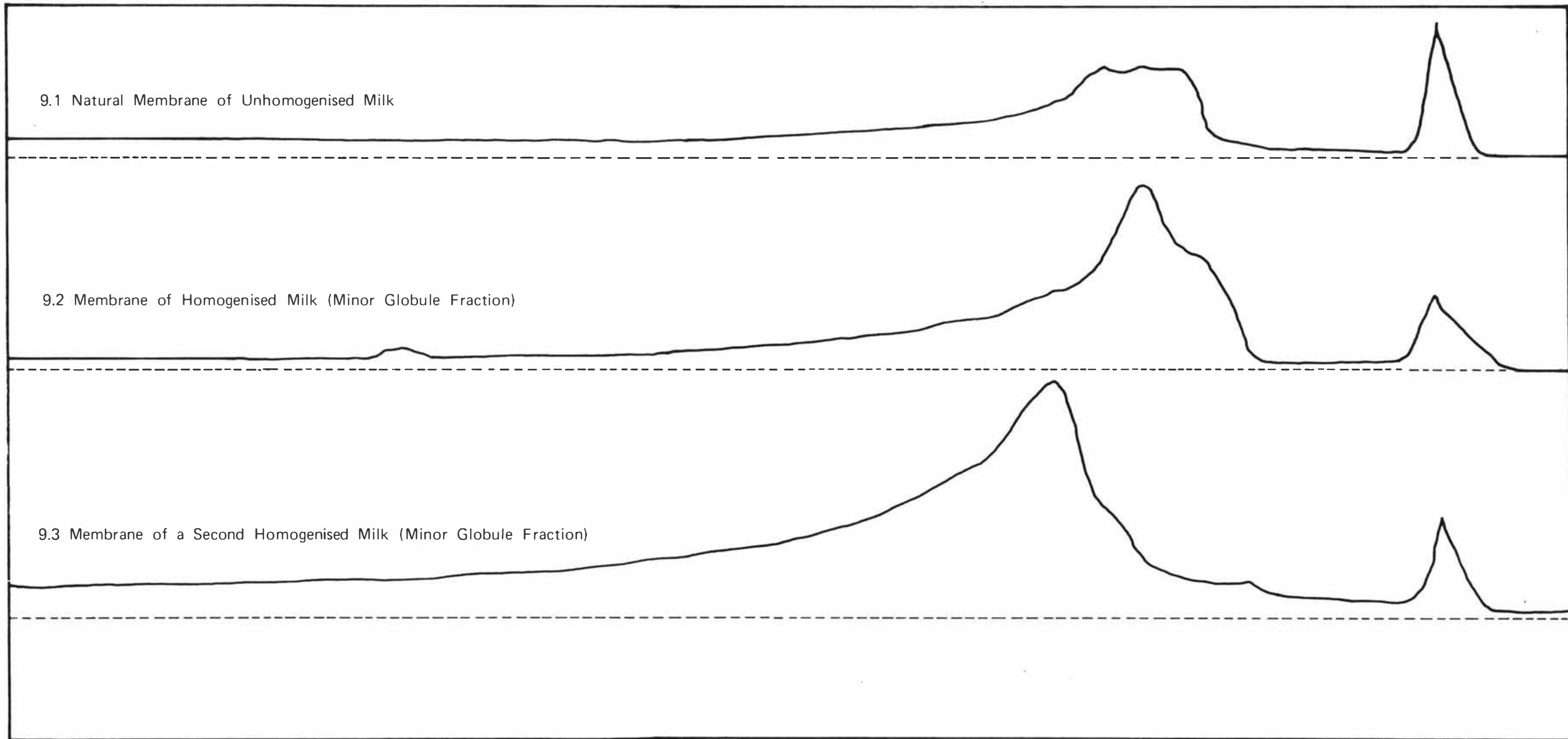
Considering the materials extracted from the small globules of homogenised milk (minor fraction) similarity is seen to exist between the patterns of Fig. 7.1 and 7.2. Considering the extent of duplication achievable these might be considered to be the same. The third pattern (7.3) is, however, very different. In all cases materials having a wide distribution of charges are apparent and also there is a consistent level of material that desorbs late in the elution pattern thereby indicating its high charge. The significance of the various peaks is indicated by the electrophoretic characteristics of fractions collected (Fig. 8.1). Clearly different proteins are present in most of the peaks checked and many are homogeneous to electrophoresis (Fig. 8.1 is a re-presentation of Fig. 3.2 where it was proposed that 11 components have been detected).

The differences in the membrane materials of Fig. 7.3 (compared to 7.2 and 7.1) are dramatic and there can be little doubt that a different material is being investigated. Some of the material desorbs rapidly (at about the bed volume of the column thereby indicating that it was not adsorbed onto the beads). Some of the material is probably present in the mercaptoethanol artifact peak yet the bulk of the material desorbs later than is apparent in the other two preparations.

Clearly also the pattern 7.4 differs from that of 7.1 though they originate from globules of different sizes in the same milk. It also differs from all of the other patterns.

The reaction of S.D.S. with natural membranes (7.5 and 7.6) produces two patterns that although not identical with one another are very similar. Superficially they are also similar to the chromatograms of 7.1 and 7.2 but the nature of materials within the peaks is quite different electrophoretically. This is demonstrated by comparison of Figs. 8.1 and 8.2. Also

FIGURE 9. CHARACTERISTICS OF N.I.D. SOLUBILISED MEMBRANES



differences in the long low profiles of highly charged materials can be seen.

In summary it appears that -

a) All the membranes are heterogeneous

b) That the three origins of membrane provide different materials

c) That three homogenised (minor) membranes differ, though this needs confirmation

d) That the membrane of large globules differs from that on small globules in the same milk.

Interpretation will be carried further after the presentation of other evidence.

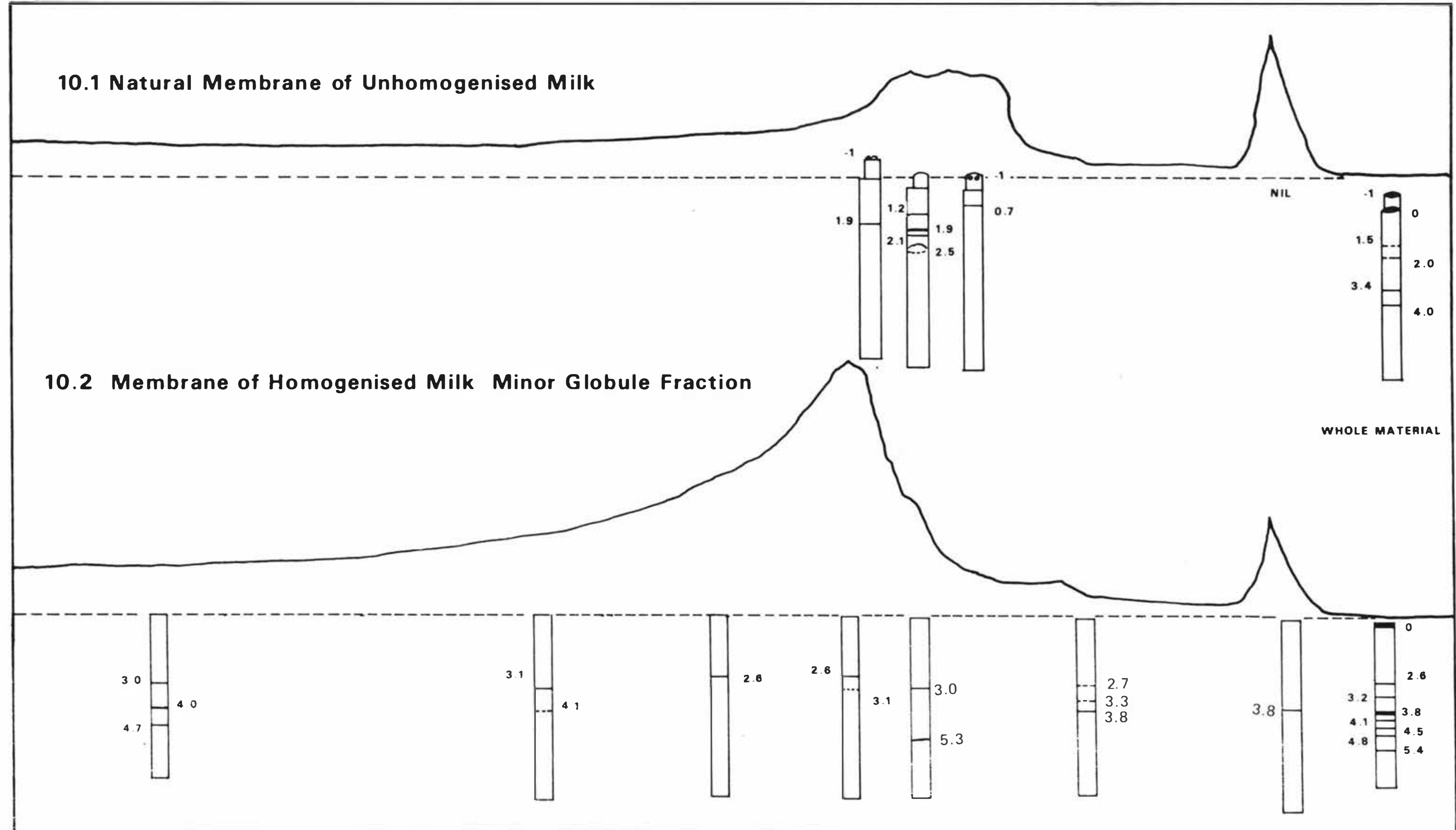
13. 2) <u>Chromatography on DEAE:cellulose of the NID Solubilised Membrane Materials</u> - Figures 9 and 10.

The materials represented in the figures are:

Fig. 9.1, 10.1, 2.1	a natural fat globule membrane of an unhomogenised milk
Fig. 9.2	a membrane material from the minor fraction of homogenised milk
Fig. 9.3, 10.2, 3.1	a membrane material from the minor fraction of another homogenised milk.

Consistent with the results obtained by the S.D.S. technique clear differences between the patterns of these materials are presented. The differences between the two homogenised milk materials (Fig. 9.2, 9.3) are distinct and there is total dissimilarity with the natural fat globule membrane material. Chromatography has not, however, provided excellent separation of the proteins as all peaks are heterogeneous but it nevertheless has value in allowing

FIGURE 10. SIGNIFICANCE OF DEAE CHROMATOGRAPHIC PATTERNS OF N.I.D. SOLUBILISED MATERIALS



fractionation and subsequent enumeration of the component materials (see page 111 discussion of Figs. 2.1 and 3.1). The natural fat globule membrane yields 6 components whereas 11 are detectable in the material of homogenised milk.

The heterogeneity of membrane materials and the differences between materials are established and the conclusions drawn from the S.D.S. patterns are supported by these results. This technique is useful but peaks obtained are heterogeneous and need study. Electrophoresis of the whole materials after NID solubilisation proved even more useful for it could be pursued more rapidly and with greater replication of results. Consequently DEAE chromatography was discontinued in favour of polyacrylamide gel electrophoresis of the NID solubilised whole membrane materials.

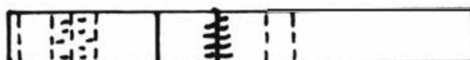
13.3) Electrophoresis of the Various Membrane Materials

Electrophoresis of the S.D.S. complexes was not particularly successful because of excessive tailing and spreading of material throughout the gel. In general, the results clearly showed dissimilarity between various materials but as results were not clear cut they are not presented except for the two patterns of Fig. K.

Figure K. Distinction Between Natural Membrane and Homogenised Milk Membrane (Minor Globule Fraction) — S.D.S./P.A.G.E.

1. Natural

2. Minor



Electrophoresis of the NID solubilised materials was much more successful though results are still not as clear as for native proteins. Such lack of clarity might be attributable to protein denaturation after surface adsorption which creates a lack of characteristic components.

The natural membrane separated into four components under the conditions of investigation and these results have already been presented (Fig. F page 105) with comment about the reproducibility of results from membranes originating from three milks. Considering the erosion of membrane materials that is inherent in the washing of the creams the similarity of these three results provides evidence for the reliability of the analytical techniques employed. Inspection of these results and comparison with those of homogenised milk (Fig. L) supports the conclusions apparent in all three approaches to the problem that the natural and homogenised membranes are very different from one another. The membranes of homogenised milk contain many more components and it is not possible to identify the original membrane components with components in the new membrane.

Electrophoretic characteristics of the NID solubilised membrane materials extracted from three homogenised milks are presented in Fig. L. Each pattern represents the results of duplicated electrophoresis. The identity of materials used was:

L.1 The membrane extracted from globules of the minor fraction of an homogenised milk

L.2 The membrane extracted from globules of the separable fraction of the same milk as L.1

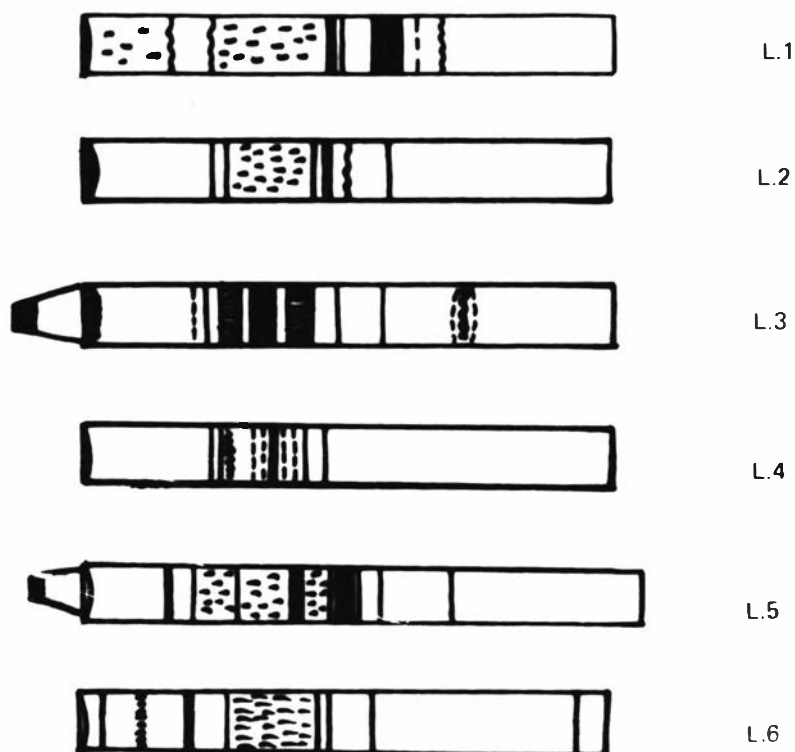
L.3 The membrane extracted from the minor fraction of a second homogenised milk

L.4 The membrane extracted from the separable fraction of the second homogenised milk

L.5 The membrane extracted from the minor fraction of a third homogenised milk

L.6 The membrane extracted from the separable fraction of the third homogenised milk.

Figure L. Comparison of Fractions of the Same Milks (N.I.D./P.A.G.E)



The heterogeneity of the membrane of the homogenised milks is confirmed particularly as the fractionation studies have shown that some of the bands are themselves heterogeneous. Consistent with the evidence of the two chromatographic approaches there are distinct differences between the three homogenised milks. Not only is this so but there are also distinct differences between the materials of the separable globules and the smaller globules of the minor fraction which accords with the same indication interpreted from the S.D.S.-complexes (Figs. 7.1 and 7.4). In all cases some material

has failed to enter the gel and this is presumably either large aggregated materials that are inadequately solubilised or component molecules that are too large to enter the gel. Denatured proteins would show this effect. In most cases a wide range of migration rates is observable and some components have migrated very rapidly for the time of electrophoresis was only 60 minutes.

In summary, all three approaches have established in common that:

a) The natural membrane and the membrane of homogenised milk differ widely from one another. No common property is observed. This lack of common property is the consequence of the intentional use of the NID solubilisation technique which deliberately excluded the majority of natural fat globule membrane material.

b) The soluble materials of membranes are heterogeneous and up to 11 components have been detected from the smaller globules plus up to 9 (possibly different) detectable from the separable globules.

c) The membranes of different homogenised milks (when comparing similar globule sizes)differ from one another.

It is further inferred but with less support (only from one or two approaches) that:

d) The small and large globules of the same homogenised milk carry membrane having different components.

e) The system of preparation is not likely to have contributed to the observed differences because natural fat globule membranes from three sources do not exhibit such wide differences and are in fact remarkably similar to one another on polyacrylamide gel electrophoresis.

f) The materials of homogenised milk contain a proportion of components that are highly charged (as indicated by DEAE chromatography of the NID solubilised materials and

the rapidity of electrophoretic migration of some of these materials). The presence of sialic acid contributes a high negative charge to the surface of the erythrocyte (Eylar, Madoff, Brady and Oncley, 1962). Milk protein materials containing sialic acid might provide one source of highly charged components on the homogenised membrane.

g) A number of components complex with S.D.S. to give highly charged complexes. The ability to complex with S.D.S. is apparently a characteristic of the materials and if so may indicate the degree of hydrophobicity of the materials. In that case some of the materials are very hydrophobic in nature which is supported by the problems of solubilisation.

Section 14

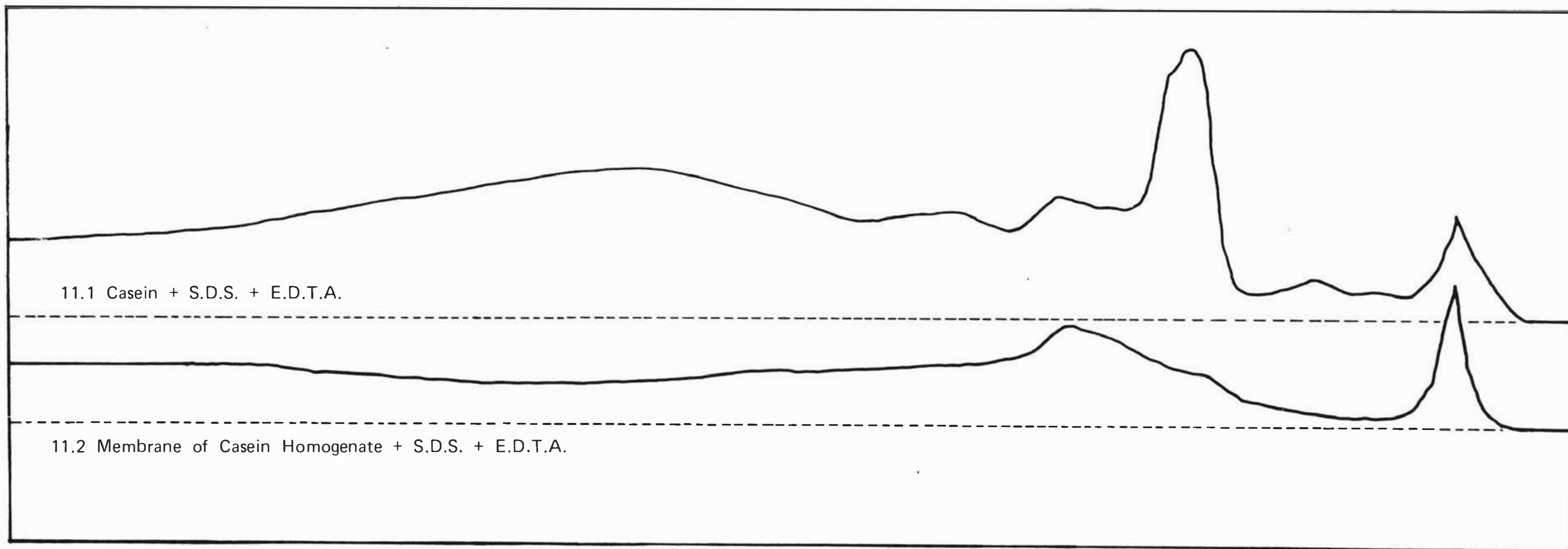
The Alteration of Native Milk Proteins by the Homogenisation Process

The characteristics of the membranes extracted from homogenised milk bear little recognisable relationship to the characteristics of native casein and whey protein. This is unexpected for a material recognised as casein by precipitation at pH 4.6 and electrophoretic properties have been detected from the membrane of homogenised milk (Jackson and Brunner, 1960). Consequently the characterisation of casein and of whey protein after adsorption onto the fat/plasma interface while independent of each other and of the natural fat globule membrane proteins has been investigated. Two homogenates were prepared; one containing casein and fat only and the other containing whey powder and fat only. The membrane materials that formed to stabilise the resultant emulsion were extracted and prepared in the same manner as for whole milk and subjected to chromatography and electrophoresis.

14.1) The Effect of Homogenisation upon Casein

A number of general observations can be made when comparing casein with its homogenate membrane. Casein is readily

FIGURE 11. EFFECT OF HOMOGENISATION UPON CASEIN (S.D.S. COMPLEXES)



soluble whereas the homogenate membrane is highly insoluble. Even in detergent solutions this insolubility is notable and during preparation a proportion of the material is centrifuged out and also a proportion of the solubilised material will still not enter the gels for electrophoresis. Changes in the electrophoretic properties of the homogenate during cold storage which indicate association, occur with the homogenate membrane whereas casein is stable to this treatment (Fig. D).

The S.D.S. complexes with casein (Fig. 11.1) and with the homogenate membrane material (Figs. 11.2 and 14.2) are distinctly different from one another on chromatography. Clearly the homogenisation process has caused a major change in the nature of the casein so that materials which separate sharply in the native state exhibit gradual desorption characteristics after homogenisation. S.D.S.:casein complexes that desorb readily from the column do not appear after homogenisation though some comparable NID solubilised components having low charge are demonstrable (Fig. 12.2).

This can be explained by assuming that casein components become modified so that they more readily associate with S.D.S. and therefore gain in charge so that they desorb less readily. This would infer that because of homogenisation the casein has become more hydrophobic -

a) So it could readily associate with the hydrophobic fat interface. This is inferred because:

b) it can readily associate with S.D.S. to form highly charged complexes

b) it loses its native solubility in water.

These assumptions infer a massive change in the conformation of casein from the stable micelle to an extended layer of heterogeneous denatured protein.

FIGURE 12. EFFECT OF HOMOGENISATION UPON CASEIN (N.I.D. SOLUBILISED)

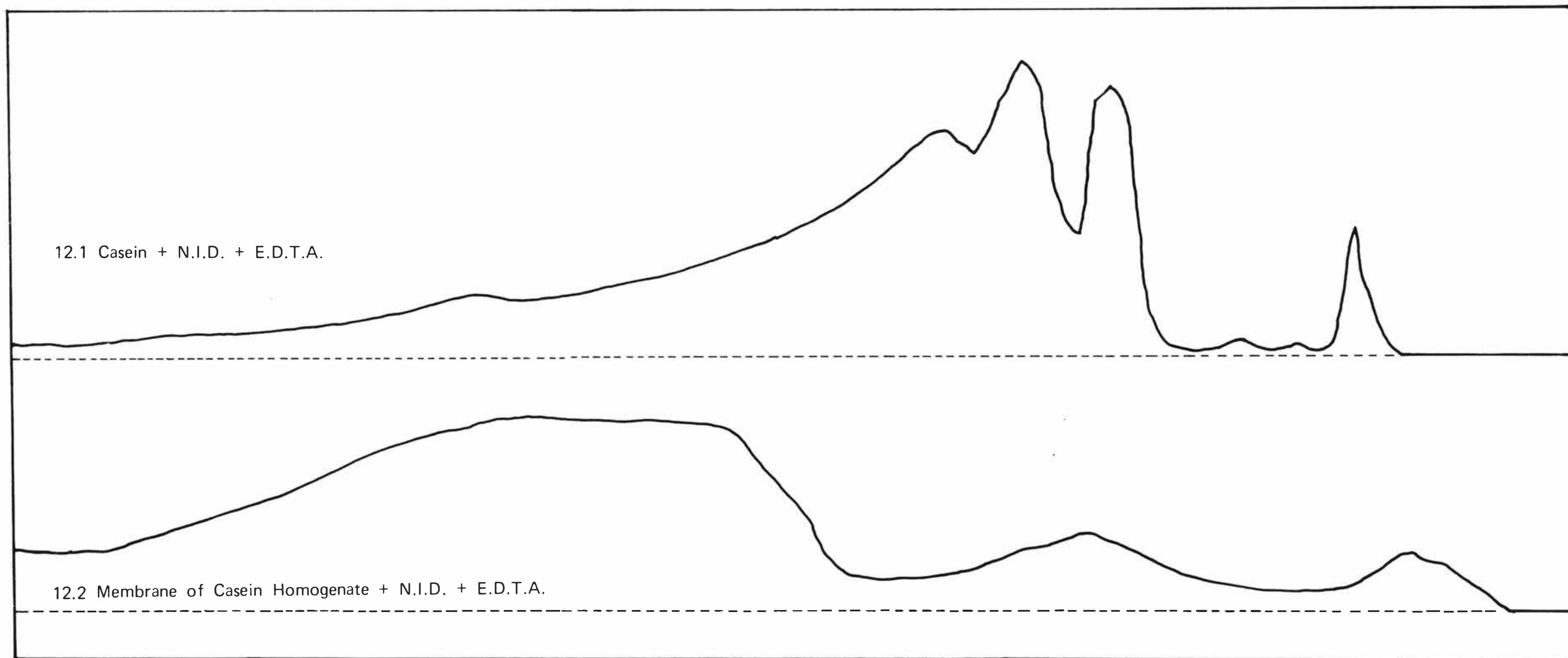
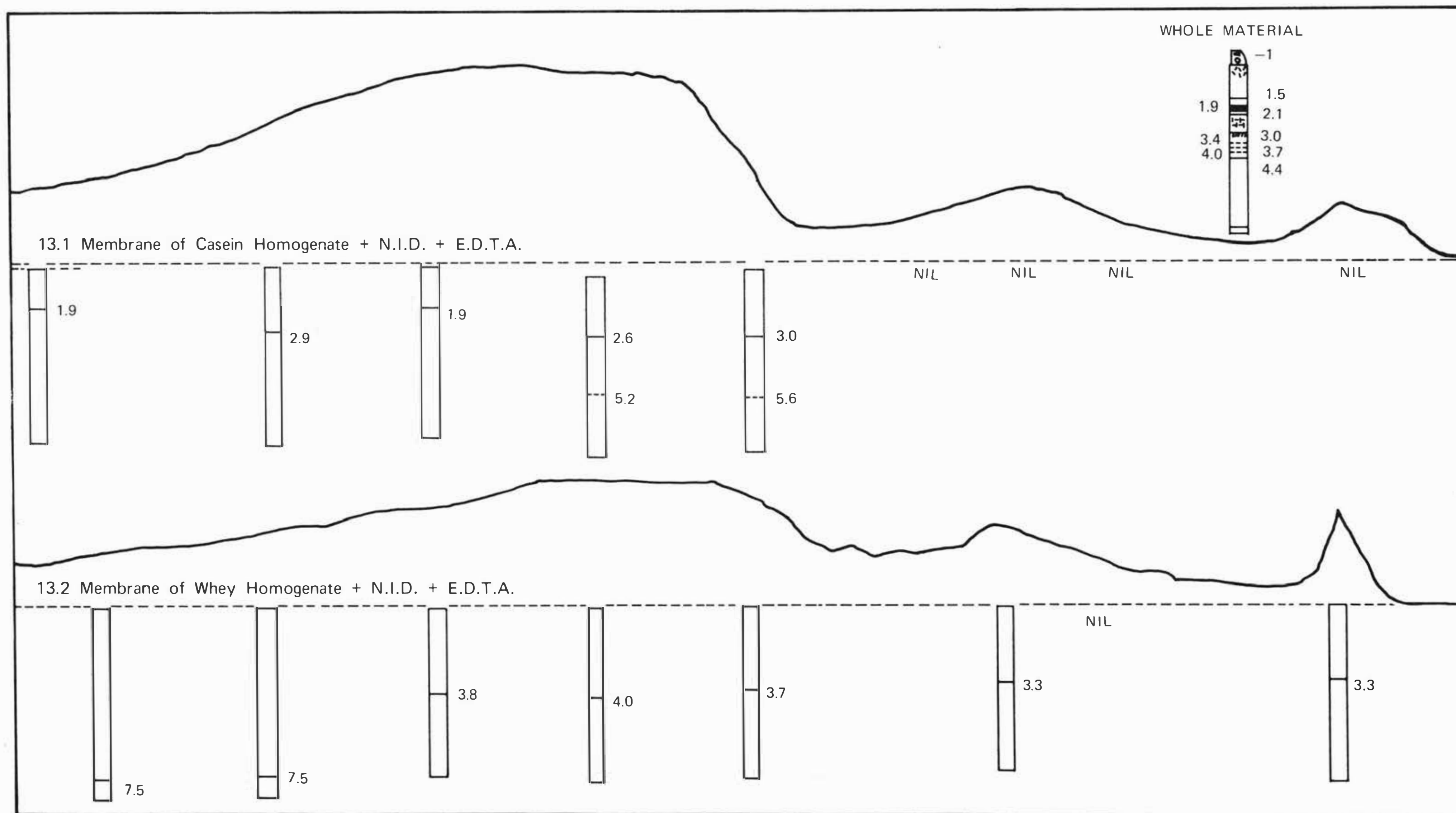


FIGURE 13. CHARACTERISTICS OF MEMBRANES OF CASEIN AND WHEY POWDER HOMOGENATES (N.I.D. SOLUBILISATION)



The massive changes that do occur are further illustrated by the character of NID solubilised casein homogenate membrane (Fig. 12.2) when compared with the characteristics of NID solubilised native casein (Fig. 12.1). Once again there are major differences in the patterns. Assuming that the NID has not contributed strongly to any change of the characteristics of the homogenate membrane components it appears that the components formed have a wide variety of charge characteristics. Some material desorbs more rapidly than the normally leading mercaptoethanol artifact peak. Interestingly, upon fractionation, the more readily desorbing material is not identifiable by polyacrylamide gel electrophoresis (Fig. 13.1) and may well be in forms that are too large to enter the gels. If so, this too distinguishes the homogenate from the native casein.

The major proportion of the homogenate membrane material is highly charged and desorbs late in the elution. Probably 5 highly charged components are detectable upon fractionation and this is not likely to represent all of the components for about 11 are detectable in the whole material. Probably more material is bound to the column and has not desorbed even at the 1.2 M sodium chloride level.

The third piece of contributing evidence is shown in Fig. M.

Figure M. Characterisation of Casein and the Membrane of its Homogenate

1. Membrane of Casein Homogenate

2. Casein

The electrophorectic characteristics of the homogenate membrane are very different from those of the native casein. Separation of the components of homogenate membrane was difficult which indicated association of the denatured materials. Interestingly, the rates of migration are not particularly high though the charges on the components of the homogenate membrane, as indicated by DEAE-cellulose chromatography, were high which suggests that the molecular dimensions of many of the components are large or extended so that migration is hindered.

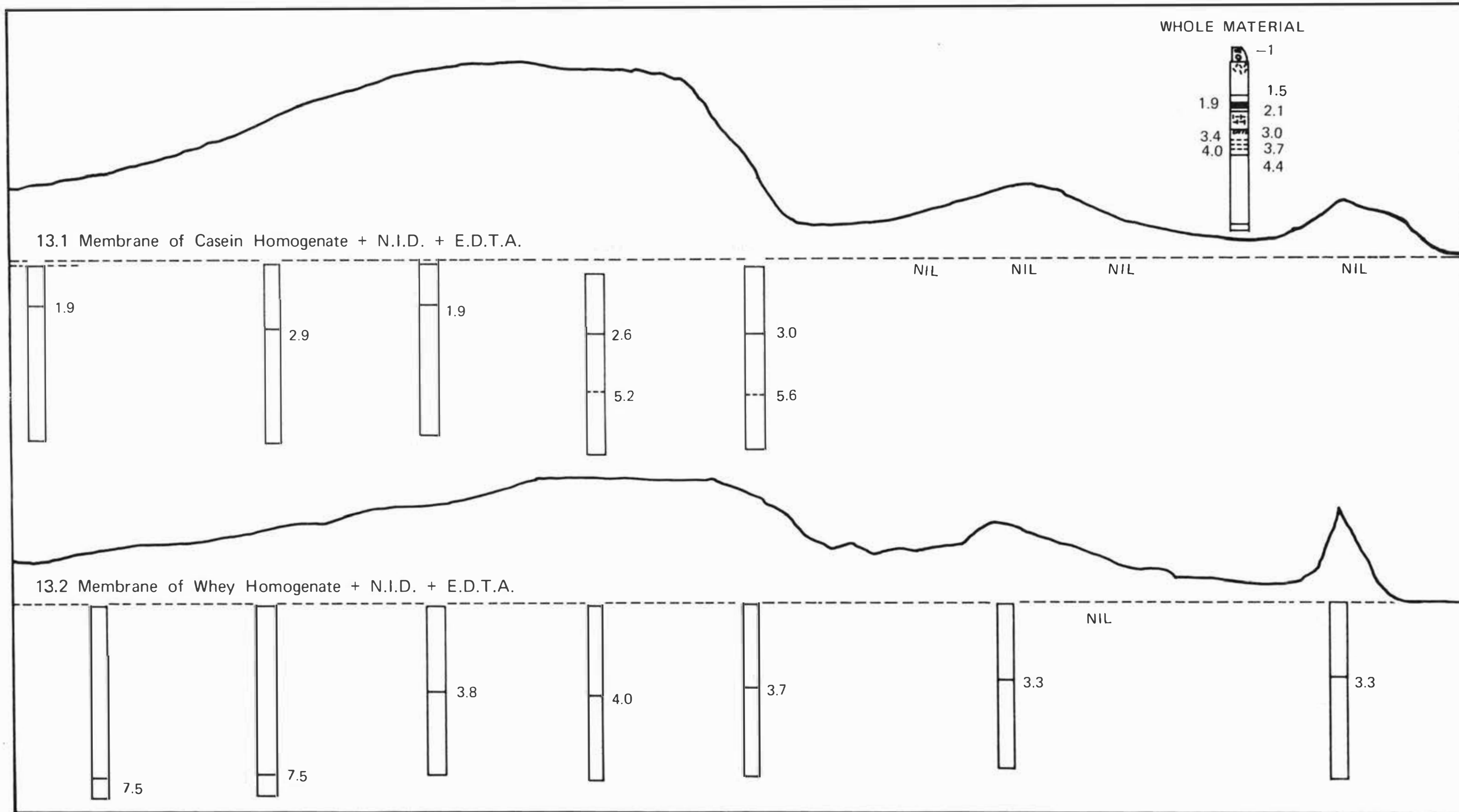
A number of conclusions can be drawn from this information

a) The casein is altered by homogenisation so that the newly formed surface predominantly consists of highly charged materials.

b) The lack of definition of the homogenate membrane components indicates a loss of character as would be consistent with massive denaturation.

c) The identity of casein after homogenisation cannot be inferred by observation of properties that appear to be the same as for casein. Previous work (see page 11) that has identified casein in the membrane of homogenised milk is, either incorrect because the techniques used to identify the materials are less selective than those used in this study, or the casein identified was loosely bound to the created membrane and was present in its native form. The electron photomicrographs of the membrane of homogenised milk show how this could occur (Henstra and Schmidt, 1970). The casein micelle in these photos appears to be either completely denatured onto the fat surface or to still retain much of its integrity as loosely held micelles attached to the globule by a portion of its surface. If this is true it is possible that the rigorous washing conditions applied in the present study might have removed all trace of native casein from the globules but left the tightly adsorbed materials. The improved selectivity of

FIGURE 13. CHARACTERISTICS OF MEMBRANES OF CASEIN AND WHEY POWDER HOMOGENATES (N.I.D. SOLUBILISATION)



the methods used in this study is favoured because the washing conditions used particularly for the separable fraction, are not very different from those normally employed by other investigators.

14.2) The Influence of Homogenisation upon Whey Protein

A homogenate of whey protein was prepared from whey powder to study the effect of the remaining components of the casein - free milk serum in case some emulsifying agents other than just the protein have an influence upon the membrane formation. Pure undenatured whey proteins in the quantities required and in the appropriate balance were not available.

In common with the observations made for casein the whey proteins have suffered major alteration because of the homogenisation process. The solubility of the proteins was again extensively reduced. The alterations are most dramatically illustrated by polyacrylamide electrophoresis (Fig. N).

Figure N. Characteristics of Whey Powder and the Membrane of its Homogenate

Solvent-treated Whey Powder in Urea (100 micro.1.)

Solvent-treated Whey Powder in Urea and N.I.D. (100 micro.l.)

Whey Homogenate Membrane in Urea and N.I.D. (250 micro.l.)

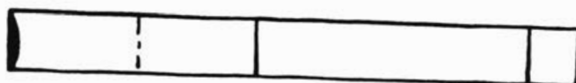
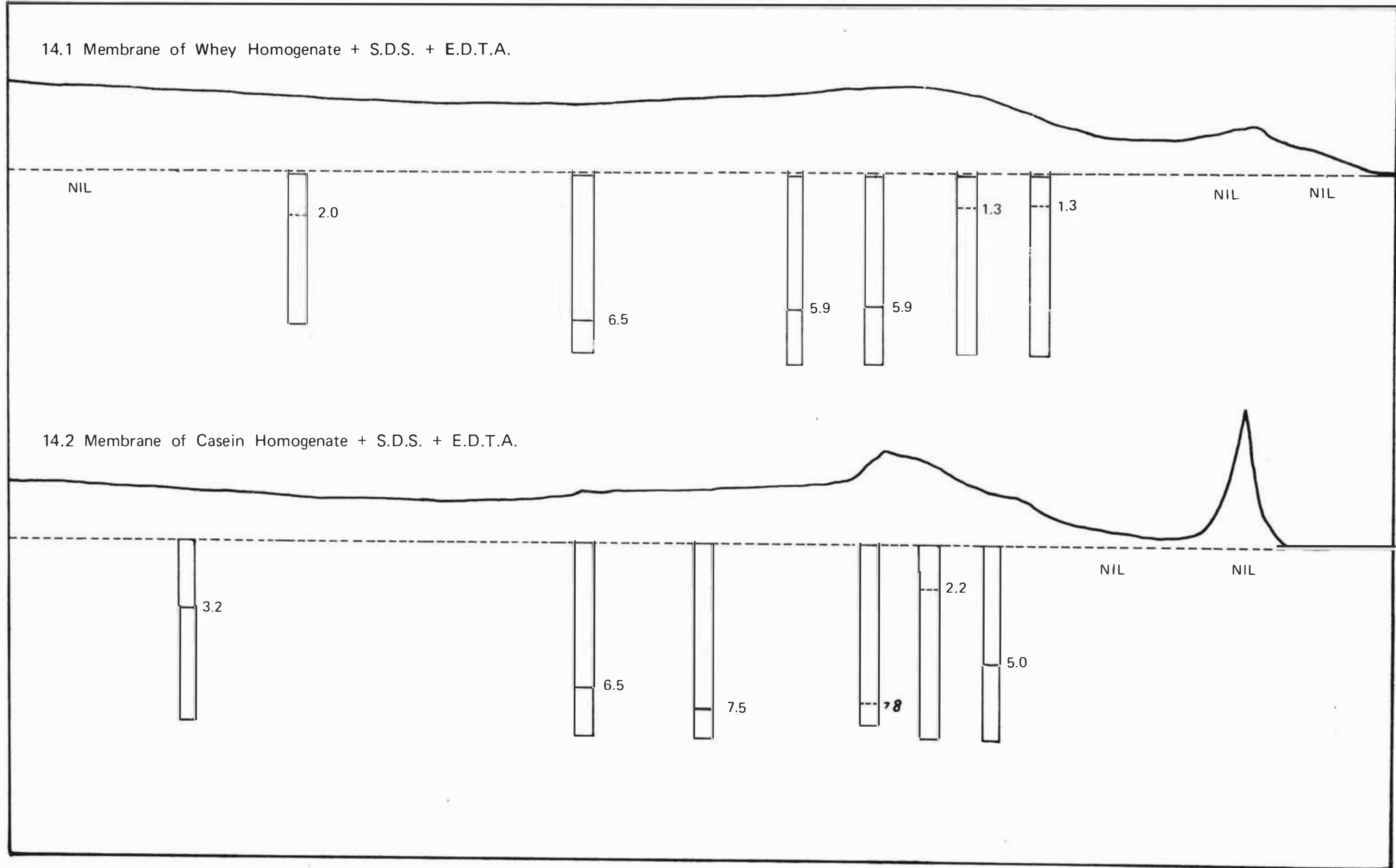


FIGURE 14. CHARACTERISTICS OF MEMBRANES OF CASEIN AND WHEY POWDER HOMOGENATES (S.D.S. COMPLEXES)



Evidently a large proportion of the material is not characterised after homogenisation (only 3 bands detectable) and thus the material bears no resemblance to that of the native material.

The whey powder, being not a pure protein material, did not lend itself to chromatography so only the homogenate membrane was characterised in this fashion (Fig. 13.2). The HID solubilised homogenate membrane contains few components identifiable by electrophoresis and again exhibits a wide range of charge characteristics. The S.D.S.-complexes are not numerous (Fig. 14.1) but again highly charged complexes are detected.

The chromatograms of the homogenate membranes of casein and whey are superficially similar but this is merely coincidental because:

i) The fractions of the HID solubilised whey and casein homogenate membranes are electrophoretically different (c/f Fig. 13.1 and 13.2)

ii) The fraction of the S.D.S.-complexes of whey and casein homogenate membranes are electrophoretically different (c/f Fig. 14.1 and 14.2)

iii) The electrophoretic patterns of the whole membrane materials differ (c/f Fig. M and N)

The investigation of the whey homogenate membrane leads to the same conclusions as for casein homogenate.
viz. After adsorption to the fat surface the whey proteins exhibit:

i) a wide variety of charge characteristics with a high proportion having high charge

ii) a wide variety of complexes with S.D.S.

iii) a major difference in electrophoretic properties from the native whey proteins

iv) a major decrease in solubility.

Also direct comparison of whey homogenate membrane and casein homogenate membrane shows that they differ from one another.

14.3) Comparison of the homogenate membranes of whey, casein, and milk

The secondary aim of preparing artificial homogenates was an attempt to identify the origin of the materials present upon the membranes of homogenised milk. It has been established that the properties of the native protein do not compare with the properties of the homogenate membranes so the "standard materials" with which to compare the properties of milk homogenate membrane must be casein and whey homogenate membranes; not the native proteins.

This may be performed by comparing electrophoretic patterns (Figs. M and N compared with Fig. L), or by comparing chromatographic patterns of the S.D.S. complexes (Fig. 14 compared with Fig. 7), or by comparing chromatographic patterns of NID solubilised membranes (Fig. 9 compared with Fig. 13).

All chromatographic patterns show a common ability of the membrane materials to combine with S.D.S. and produce complexes with high charge that show a typical upward slope. They are difficult to solubilise in the absence of detergent. These properties are shared with the natural fat globule membrane but not with the native milk proteins and so are

presumably characteristic of the materials adsorbed at the fat/plasma interface. They probably indicate highly hydrophobic properties.

A number of component materials that exhibit high charge and are detectable by polyacrylamide gel electrophoresis, when solubilised by non-ionic detergent, are demonstrable in all homogenate membranes but this is not true for natural membrane nor for native proteins (highly charged material is present in the natural membrane but it does not show a band on electrophoresis).

In other respects the artificial homogenate membranes differ markedly from the milk homogenate membrane, which is to be expected for the milk homogenate membranes all differ from one another. The number of components detectable in fractions from the chromatograms of the casein and whey homogenate membranes is lower than the number detectable from milk homogenate membranes. Possibly this reflects the greater number of source proteins in the milk. One remarkable point of difference is the chromatographic characteristics of the NID solubilised casein homogenate membrane which carries a high proportion of highly charged materials.

A number of experimental reasons may influence this reasoning and possibly differences may not be real because:

a) in milk, casein is not separated from whey components and the natural fat globule membrane components. The possibility of complexes forming between these source proteins must be recognised.

b) the phospholipids and polar lipids of milk fat would assist emulsification in milk homogenisation but would not be present in the artificial homogenates.

c) the experimental homogenisation was not as effectively performed as it was in the commercially operated

homogeniser used on the milk.

Despite these reservations it is clear that the components adsorbed to the fat:plasma interface due to homogenisation cannot be identified by reference to the properties of native casein and whey proteins.

Recognition of this factor has proved a difficulty for previous workers in this field. Free boundary electrophoresis did not permit identification of these materials in one study (Brunner, Lillevick, Trout and Duncan, 1953) but provisional identification of these materials was made on the basis of the sedimentation characteristics of native proteins (Brunner, Duncan, Trout and MacKenzie, 1953). In another study a "casein complex" was identified by reference to the free boundary electrophoretic characteristics of native casein and to the iso-electric precipitation of this material at pH 4.6. The complex, however, contained 8.5% lipid while rennet and urea had no effect upon it (Jackson and Brunner, 1960). These last observations accord with those of the present study that casein's native properties are lost after homogenisation so the conclusion drawn, that the complex was casein must be open to doubt. The preparation of an homogenate of calcium caseinate, washed membrane and butteroil appeared to be a more valid approach (Jackson and Brunner, 1960). The use of free boundary electrophoresis to identify the complex possibly invalidated the identification because of the insensitivity of the technique. Similarly, a comparability between the charge charactersitics of whey and casein homogenate membranes (as detected by chromatography) was also noticeable in the present study but was shown to be invalid by more sensitive techniques. Identification of the whey proteins after homogenisation was attempted but not achieved (Jackson and Brunner, 1960) and this is in conformity with the results of the present study.

In summary, it appears that the alteration to the nature of the proteins of milk caused by homogenisation is so extensive and the nature of the membrane of homogenised milk is so variable that identification of the origin of the freshly adsorbed materials is not feasible.

A View of the Homogenisation Process

The results presented lead to a number of postulations regarding the nature of the materials adsorbed to the fat: protein interface of milk as a result of the homogenisation process.

It is observed that homogenisation and subsequent surface adsorption causes a massive change in the characteristics of the native milk proteins so that the source of the adsorbed materials is not identifiable by comparison with the properties of the native materials. This observation invalidates many of the conclusions drawn by previous workers as to the identity of source materials that adsorb to the interface. It is, however, possible to re-interpret early results in terms of the difficulties encountered and the differences observed and find support for the conclusions of the present study.

There is no evidence that adsorption is specific to any protein fraction. The homogenisation process creates a proportion of materials that are more highly hydrophobic and more highly charged than the native source proteins. These two properties would be consistent with an improvement in their properties as emulsifying agents which is a part of their new function. Similarly the proteins of the natural fat globule membrane are no longer identifiable in the membrane material of homogenised milk. Because of the greater surface area of globules of homogenised milk the natural membrane components would be diluted by other materials but neither of these facts exclude the probability of their presence in the new membrane, albeit in an altered form.

The process of adsorption is apparently unordered for the materials created differ among milks and even between globule sizes. It must therefore be assumed that the origin of

materials that are adsorbed is not specific though possibly protein materials that act as emulsifying agents (or which are most readily converted to emulsifying agents) might be preferred by the environment of the freshly formed fat:serum interface. Conceivably the proteins are spread over the surface of the globules after an unfolding of the original structure in a manner similar to that which occurs at other interfaces.

In view of these considerations it must be inferred from the interfacial activity of the various milk proteins that layers containing mixtures of the remnants of natural globule membrane proteins, whey proteins, and caseins will occur. The variability observed probably reflects the importance of the environment of the interface upon the form that adsorption will take.

The energy required to perform these effects will be a composite of mechanical and surface energies. Variation of mechanical energy would act to alter globule size and consequently surface area. Because mechanical energy is effective in altering the properties of skim milk it probably can affect the protein stability directly. Proteins are also probably more susceptible to interfacial adsorption after denaturation. Since casein in micellar form has little surface activity mechanical disruption and/or distortion of the micelles would assist the surface adsorption of casein.

The consequences of this process are that protein stability of the homogenised milk is likely to be reduced and that the reactivity of the fat is likely to be altered because of the new covering layer of denatured protein on the globule surface. Such effects have been widely observed in the technological literature from which a number of inferences can be drawn to complete this view of the homogenisation

process. These are:

1) Because of the massive changes of protein properties as observed it is likely that lipase would be inactivated if it were incorporated within the homogenisation-created membrane. Its action is therefore probably of independent adsorption subsequent to the initial resurfacing phenomena.

2) The reduction of protein stability may occur because of the denaturation of a proportion of the protein through adsorption, through its involvement in the creation of dense fat:protein complexes, through direct alteration arising from mechanical forces, or some combination of these effects.

3) The envelope of denatured material may well protect fat against oxidation by binding less copper, by lowering the concentration of phosphatide at the surface, and/or removal of lipo-protein units from the surface.

4) The incorporation of a component of fat globule agglutinating factors into the created membrane could destroy its ability to promote globule clustering though denaturation of the factor might also have occurred merely as a consequence of the violence associated with the process.

5) The protection of the milk fat emulsion against freezing damage is explainable on the assumption that the enveloping membrane is so denatured as to be insensitive to further denaturation. This could also contribute to the stability of homogenised globules to churning.

6) The denatured protein envelope acts as a good emulsifying agent so that the reduced concentration of phosphatides

at the surface does not result in emulsion instability.

7) The limited capacity of protein to stabilise the emulsion of high fat content is understandable because an 80% fat cream would contain only about 0.6% protein which could hardly provide an enveloping membrane around the many fat globules.

8) The salt balance of milk need not be measurably altered to establish the complex provided hydrophobic fat/protein associations are assumed to be the prime bonding agents. If this is true the value of EDTA in dispersing the membranes must be restricted to its value in dispersing naturally occurring linkages within the natural membrane and intra-casein linkages.

In summary it is proposed that the primary action of the homogenisation process is to create an area of exposed fat/liquid interface on to which surface denaturation of the proteins of milk can occur. This results in massive alteration of those proteins that become tightly bound, so that they are no longer identifiable by comparison with the properties of the native proteins. The actual form that is taken by such adsorption is controlled by factors relating to the fat/liquid interface such as surface pressure, and to the nature, concentration and availability of all surface active materials that are present in its environment. This results in variability in the nature of the materials that can be isolated from membranes which originate from different milks or which originate from globules of different sizes within the same milk. This variability suggests that preferential adsorption of any one protein does not occur to any measurable extent.

As the properties of skim milk can be altered by the homogenisation process it appears that a secondary action of

homogenisation is an alteration of protein structure which may promote the surface adsorption phenomena.

The total effect is to create around the newly formed fat globules a shell of denatured protein material which has properties that are distinct from those of the original membrane and are also distinct from those of the native proteins of milk. Consequent upon these changes some of the properties of milk can be observed to have altered.

APPENDIX 1

Calculation of diameter of "cut-point" particle

1. **For the Alfa-Laval Separator**

It is given that $Q = 2\ V_g \Sigma$ (Equation 1)

where Q = flow rate through separator,

Vg = terminal sedimentation velocity of the globule,

Σ = the area of a gravity settling tank of equivalent sedimentation characteristics to the centrifuge.

For a disc bowl separator equation 17 gives:

$$\Sigma = Kr\ \frac{2\pi\ n\omega^2\ (r_2^3 - r_1^3)}{3\ g\ \tan\theta}$$

where Kr = a constant related to class of a centrifuge and has been shown to be 0.5 for a disc bowl separator,

n = number of discs in bowl = 40,

ω = angular velocity, radians/sec = 2 revs/sec = 2 x 133,

r_2 = radius to periphery of disc stack = 7 cm,

r_1 = radius to internal point of disc stack = 2 cm,

θ = the angle to the vertical at which the discs rest = 40°.

Hence

$$\Sigma\ (\text{disc bowl}) = 0.5 \times \frac{2\pi \times 40 \times (2\pi \times 133)^2 \times (7^3 - 2^3)}{3 \times 981 \times \tan 40^\circ}$$

$$= 11.90 \times 10^6$$

Now $Q = 2\ V_g\ \Sigma$ Eq. 1

where Q = flow rate = 100 ml/second

$$\therefore \quad V_g = \frac{Q}{2\Sigma}$$

$$= \frac{100}{2 \times 11.9 \times 10^6}$$

$$= 0.420 \times 10^{-5}$$

It is given that $V_g = \dfrac{\Delta\rho\, D^2\, g}{18\,\eta}$ Eq. 3 which is a statement of Stoke's Law

$$\therefore \quad D^2 = \frac{V_g\, 18\,\eta}{\Delta\rho\, g}$$

where D = diameter of the cut point particle,
= the diameter of particles half of which will be removed in passage through the centrifuge bowl and half of which will not be removed,

See McDowall (1953):
η = viscosity of the milk = 0.010 poise at 40°C,
$\Delta\rho$ = difference in density between fat and milk serum = 0.122 gm/ml at 40°C.

Hence $$D^2 = \frac{0.420 \times 10^{-5} \times 18 \times 0.01}{0.122 \times 981}$$

$$= 63.5 \times 10^{-10}$$

$$\therefore \quad D = 7.97 \times 10^{-5}\ \text{cm}$$

$$0.8\,\mu$$

From this calculation it may be concluded that the Alfa-Laval separator used will satisfactorily remove globules from the homogenised milk that are larger than the average size. This is in satisfactory agreement with the observed fat recovery.

2. Use of the Sharples Supercentrifuge

The Sigma Value may be calculated as follows:-

$$\Sigma = \frac{V\omega^2}{g} \quad \frac{1}{\ln\left(\dfrac{2\,r_2^{\,2}}{r_2^{\,2} + r_1^{\,2}}\right)} \qquad \text{Equation 11}$$

where V = Volume of liquid in the bowl

= 230.8 ml

ω = Angular velocity, radians/sec

= 2π revs/sec.

= $2\pi \frac{40,000}{60}$ = 2 x 666

NB Machine runs most effectively using homogenised milk as feed with dam no. 7.5 at 40,000 r.p.m. with an inflow rate of 8.8 ml/sec.

r_1 = internal radius of column of milk

= internal radius of cream overflow dam. = 1.07 cms

r_2 = internal radius of centrifuge bowl

= 2.219 cms

$$\text{Hence } \Sigma = \frac{230.8 \times (2\pi\ 666)^2}{981} \quad \frac{1}{\ln\left(\frac{2 \times 2.219^2}{2.219^2 + 1.07^2}\right)}$$

$$= 8.54 \times 10^6$$

$$\text{Now } V_g = \frac{Q}{2\Sigma}$$

$$= \frac{8.8}{2 \times 8.54 \times 10^6}$$

$$= 0.515 \times 10^{-6}$$

$$\text{Now } D^2 = \frac{V_g\ 18\ \eta}{\Delta\rho\ g}$$

where values are the same as before.

$$= \frac{0.515 \times 10^{-6} \times 18 \times 0.010}{0.122 \times 981}$$

$$= 7.79 \times 10^{-10}$$

$$\therefore D = 2.79 \times 10^{-5} \text{ cm}$$

$$\simeq 0.3 \text{ microns}$$

From this it might be concluded that useful separation of the fat, including the smaller than average diameter fat globules from homogenised milk could be effected. In

practice however this was not found to be so. Separation of a fraction of homogenised milk containing 1.3% fat produced a cream containing 2.8% fat, whilst 1.1% fat appeared in the skim milk.

(Note:- The operation of the machine could theoretically be improved by reducing the radius of the ring dam in use. This procedure would however increase the proportion of liquid flowing to the cream outlet thereby reducing the test of the cream even further. If a larger ring dam was used the cream test would improve because a lower volume of liquid would pass to the cream outlet but the fat content of the effluent "skim" milk would still be unacceptably high (around 1.5%).

Because of these factors it was necessary to abandon the use of continuous centrifuging techniques to obtain the small diameter globule fraction.

3. The Use of a Bucket-type MSE Centrifuge

The diameter of the cut-point particle may, for a bucket centrifuge, be calculated more directly from the following formula:

$$t = \frac{18}{\Delta\rho D^2 \omega^2} \ln \frac{r_2}{r_1} \qquad \text{No. 8}$$

$$\text{or} \quad D^2 = \frac{18}{\Delta\rho t \omega^2} \ln \frac{r_2}{r_1}$$

where t = time of centrifuging (but ignoring speed-up and slow-down times)

= 30 mins = 30 x 60 secs.

ω = radians travelled per second

= 2π revs/sec

= $2\pi \frac{5000}{60}$

r_2 = radius to outer wall of centrifuge bottle from centre of rotation

= 13.3 cms

r_1 = radius to inner wall of centrifuge bottle from centre of rotation

= 5.022 cm.

(Note:- In arriving at values for r_1 and r_2 the angle at which the bottles lie in the centrifuge head must be considered.)

(Note:- The value for $\Delta\rho = 0.122$ may be in error due to the protein membrane which is proportionally more important, altering the effective density of the fat.)

$$\text{Hence } D^2 = 18 \times \frac{0.01}{0.122} \times \frac{1}{30 \times 60} \times \frac{1}{\left(2\pi \frac{5000}{60}\right)^2} \ln \frac{13.3}{5.022}$$

$$= 30.36 \times 10^{-12}$$

$$\therefore \quad D = 5.510 \times 10^{-6}$$

$$= 0.055 \text{ micron.}$$

This result infers that these conditions of centrifugation will significantly improve separation.

The nett result is:-

(a) The homogenised milk can be separated in the Alfa-Laval separator to produce a cream fraction containing approximately half of the fat which exists in globules that are unlikely to be less than 0.8 micron in diameter.

(b) The serum from this can be re-centrifuged batchwise in an MSE centrifuge at 5000 r.p.m. for 30 mins. to provide a 2nd fraction containing globules of smaller size.

APPENDIX 2

Determination of Protein - Kjeldahl Nitrogen Determination

Weigh out accurately an appropriate quantity of material. Insert filter paper and sample into a Kjeldahl flask.

Digestion

Add about 2 gms of Na_2SO_4 to raise the boiling point, 5 mls of mercuric sulphate as a catalyst, and 20 mls of conc. H_2SO_4. Also add two glass beads to prevent bumping. Heat gently in a fume cupboard. When frothing ceases turn up heat and boil gently until solution is clear. Cool, wash down neck of flask with hot distilled water and heat to fumes of H_2SO_4. Cool, dissolve precipitate in distilled water. Transfer quantitatively to 100 ml volumetric flask, and dilute to mark using dilution water to rinse out Kjeldahl flask thoroughly. Mix, readjust to volume if necessary.

Distillation

Transfer 10 ml aliquot to the distillation flask, assemble the apparatus and run in 15 ml of 60% $NaOH/Na_2S_2O_3$ solution.
(Note:- Prepare this as follows:-

60 gm NaOH and 5 gm $Na_2S_2O_3$ to 100 ml with water)

(Alternatively:- 15 ml of 60% NaOH and 5 ml of 15% sodium hypophosphite may be used as there is a danger of the formation of H_2S being produced when using thiosulphate.)

Steam distil on micro-distillation apparatus for 15 minutes, collecting distillate in 5 mls saturated (4%) boric acid, plus 5 ml water.

Titrate boric solution with 0.02 N HCl, using Tosheros indicator to gray/green end point, or first appearance of violet. (Indicator is 2 parts 0.2% methyl red in ethanol with 1 part 0.2% methylene blue in ethanol).

APPENDIX 3

Conventions employed in Presentation of graphical results

The graph lines drawn by the recorder have been traced and reduced to half size. The X axis represents the increasing gradient of sodium chloride with time and is read from right to left. Hence in the lowest tracing of figure 1 the experiment begins slightly before position A with all of the protein bound to the DEAE sephadex beads and no salt in the buffer.

Progressively the salt concentration in the buffer increases as the graph line moves to the left. The first protein is desorbed at the salt concentration reached at position C and all of the protein has desorbed before position D. The increase in salt concentration continues until it reaches 1.2 Molar at position E after an elution period of 16 hours.

The Y axis is a scale of optical density as measured by the absorption of light at 280 mM. As each protein passes through the cell it is measured at 3 minute intervals and a record of the O.D. is made. Each peak relates to the concentration reached at that time. The peak position B is present in every graph and represents a reference point to which all the graphs are aligned for comparative purposes. It is not considered to be a true protein peak. A horizontal dotted line is included to indicate the base line.

In Figure 2 the graphs are complemented by a characterisation of the protein material in each peak as shown by polyacrylamide gel electrophoresis. The pattern at the far right shows the nature of the whole membrane. The direction of migration is downwards. The proteins enter the large pore gel at position -1 and enter the small pore gel at position 0 which

is designated as the origin. The bands of protein are drawn as observed with dotted lines representing faint or curved lines, and shaded areas represent broad bands of protein. Dark lines at the origin or at -1 represent protein material that is insufficiently dissociated, or naturally too large, to enter the gel at that point. The numbers written beside each protein band represent the distance of migration in centimetres achieved after 60 minutes of electrophoresis.

The term "protein-free" means that no protein is detectable. The term "Nil" means that while the U.V. absorption pattern of fractions collected at that point indicate the presence of protein no characteristic protein band appears on electrophoresis. This term is not used unless duplicate, or more often triplicate, electrophoretic runs have failed to detect protein. It could mean that:

a) the protein material is in low concentration or is diffused along the gel so that the stained material is not visible,

or

b) the protein material is in too large a particle form to enter the gels.

In the figures where the polyacrylamide gel is drawn horizontally the direction of migration is from left to right.

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Abbreviations used are, wherever possible, the same as those listed in "World List of Scientific Periodicals" 4th Edition.