

THE LIMITING THICKNESS OF FREE PROTEIN FILMS:

A STUDY OF PROTEIN FOAM LAMELLAE

A THESIS

presented by

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ABSTRACT

The limiting thickness of free aqueous protein films has been measured for bovine serum albumin, bovine- γ -globulin, pepsin, lysozyme, insulin, α -casein, β -casein, κ -casein and ovalbumin. Attempts have also been made to elucidate the mode of adsorption of proteins by studying synthetic polypeptides. The results on free films for the more stable protein solutions have been correlated with observations on bulk foaming properties.

Bovine serum albumin was chosen for the main study because the physical parameters necessary for analysis of the results are either readily available or may be estimated with confidence. Electrostatic forces do not control the thickness of albumin films and it is postulated that short range forces of repulsion stop the surfaces from approaching each other. The limiting thickness is then controlled by the amount of stretching allowed by the adsorbed surface layers as they deform from the hemispherical shape.

When the electrical double layers are depressed by 0.1 N KCl the limiting thickness does not correspond to either two surface layers of native proteins or two 'fully spread' surface layers each about $10 \overset{\circ}{\text{A}}$ thick. It is postulated that serum albumin can only partly unravel and the unravelling is controlled by the disulphide bonds in the molecule. This has been justified by reducing the S—S bonds and

observing different characteristics for films formed from solutions of reduced albumin. The surface layers of albumin solutions have been shown to contain molecules in different molecular configurations.

The limiting thickness of films stabilised by other proteins were greater than for albumin even in 0.1 N KCl. These proteins have also been postulated to contain molecules in different configurations, some of which are subject to 'surface coagulation'. All of the proteins studied could produce films of uneven thickness and in general the conditions for high stability of protein foams corresponded with those for producing such films.

Attempts to elucidate the mechanism of protein adsorption by using commercially available synthetic polypeptides failed because the delicate balance of hydrophilic to hydrophobic groups combined with aqueous solubility could not be matched.

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TABLE I

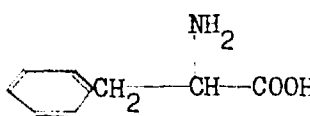
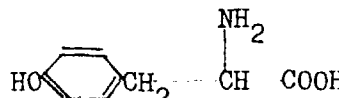
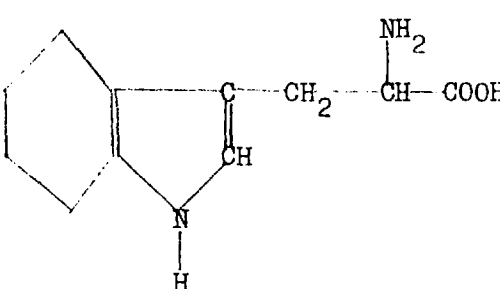
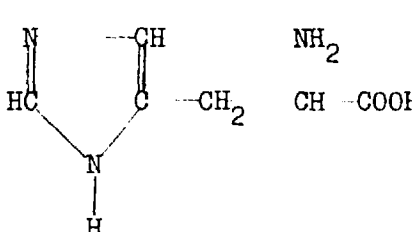
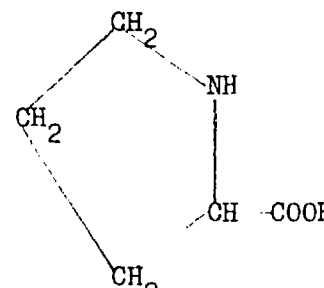
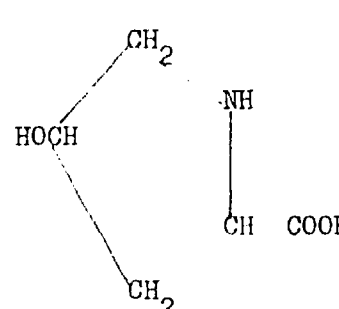
A M I N O A C I D S

Common Name	Code Name	Iso- Electric Point	Formula
Glycine	Gly	6.0	$\begin{array}{c} \text{NH}_2 \\ \\ \text{H} - \text{CH} - \text{COOH} \end{array}$
Alanine	Ala	6.0	$\begin{array}{c} \text{NH}_2 \\ \\ \text{CH}_3 - \text{CH} - \text{COOH} \end{array}$
Valine	Val	6.0	$\begin{array}{c} \text{CH}_3 \quad \text{NH}_2 \\ \quad \\ \text{CH}_3 - \text{CH} - \text{CH} - \text{COOH} \end{array}$
Leucine	Leu	6.0	$\begin{array}{c} \text{CH}_3 \quad \text{NH}_2 \\ \quad \\ \text{CH}_3 - \text{CH} - \text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Isoleucine	Ileu	6.0	$\begin{array}{c} \text{CH}_3 \quad \text{NH}_2 \\ \quad \\ \text{CH}_3 - \text{CH}_2 - \text{CH} - \text{CH} - \text{COOH} \end{array}$
Serine	Ser	5.7	$\begin{array}{c} \text{OH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Threonine	Thr	5.6	$\begin{array}{c} \text{OH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_3 - \text{CH} - \text{CH} - \text{COOH} \end{array}$
Aspartic Acid	Asp	2.8	$\begin{array}{c} \text{COOH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Asparagine	Asn	-	$\begin{array}{c} \text{CONH}_2 \quad \text{NH}_2 \\ \quad \\ \text{CH}_2 - \text{CH} - \text{COOH} \end{array}$

A M I N O A C I D S

Common Name	Code Name	Iso-Electric Point	Formula
Glutamic Acid	Glu	3.2	$\begin{array}{c} \text{COOH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Glutamine	Gln	-	$\begin{array}{c} \text{CONH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Lysine	Lys	10.0	$\begin{array}{c} \text{NH}_2 \quad \text{NH}_2 \\ \quad \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Hydroxylysine	-	-	$\begin{array}{c} \text{NH}_2\text{OH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Arginine	Arg	10.8	$\begin{array}{c} \text{NH}_2 \quad \text{NH}_2 \\ \quad \\ \text{CNHCH}_2\text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \\ \\ \text{NH} \end{array}$
Cysteine	CySH	5.1	$\begin{array}{c} \text{SH} \quad \text{NH}_2 \\ \quad \\ \text{CH}_2 - \text{CH} - \text{COOH} \end{array}$
Cystine	CySSCy	4.8	$\begin{array}{c} \text{NH}_2 \\ \\ \text{S} - \text{CH}_2 - \text{CH} - \text{COOH} \\ \quad \\ \text{S} \quad \text{CH}_2 - \text{CH} - \text{COOH} \\ \\ \text{NH}_2 \end{array}$
Methionine	Met	5.7	$\begin{array}{c} \text{S} \quad \text{CH}_3 \quad \text{NH}_2 \\ \quad \quad \\ \text{CH}_2\text{CH}_2 - \text{CH} - \text{COOH} \end{array}$

A M I N O A C I D S

Common Name	Code Name	Iso-Electric Point	Formula
Phenylalanine	Phe	5.5	
Tyrosine	Tyr	5.7	
Tryptophan	Try	5.9	
Histidine	His	7.6	
Proline	Pro	6.3	
Hydroxyproline	Hypro	5.8	

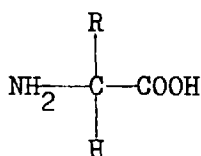
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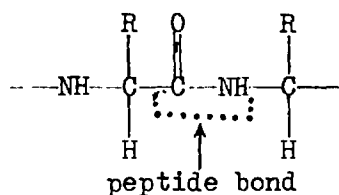
CHAPTER 1. INTRODUCTION

A. The Proteins

The Proteins are a group of substances found in nature which are essential ingredients for living matter. All growth and reproductive mechanisms involve proteins, and many biological degradation reactions involve enzymes (which are proteins). Proteins consist of polyamino acid chains, the polymeric link being the -peptide bond (fig 1).



amino acid



protein

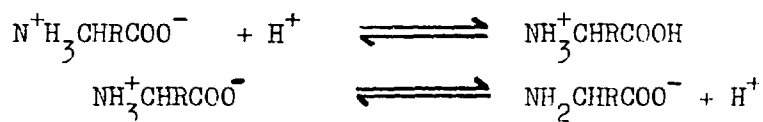
fig. 1.

Most proteins contain some of each of the commonly occurring amino-acids. Of the eighty or so known amino-acids, about twenty occur frequently in proteins and these are listed in Table I, together with their side chains, their accepted abbreviations and iso-electric points. The one exception in the classification of amino-acids is

proline which has an imino ($-N-H-$) instead of an amino ($-NH_2$) group. The remainder differ exclusively in their side chains (R). Certain proteins (e.g. ovalbumin, γ -globulin, κ -casein) contain carbo-hydrate in the molecule which appears to be covalently bound. However, this is little understood and will not be discussed further.

Examination of Table I shows that proteins possess in their structure the elements of "amphipathic" nature, in the surface chemical sense, i.e. some portions of the molecule are lipophilic and others hydrophilic, and it is this that confers individuality on the surface chemistry of the different proteins. Indeed, it turns out that the balance between these portions has a direct bearing on most of the properties of proteins in solution as well. Lipophilic properties are conferred mainly by the side chains of ala, val, leu, ileu, pro, met and phe. Hydrophilic properties are associated with the free ionisable groups on asp, glu, lys and arg, which are not in the peptide backbone, and also with the terminal groups of the various chains. Other polar but non-ionic groups such as free OH-groups of ser, thre, tyr and the NH of histidine have some binding capacity for water. The presence of ionisable groups of both charges gives all proteins amphoteric properties and it becomes necessary to decide whether the molecules

are zwitterionic (dipolar) in a manner similar to the free amino-acids. The latter are well established to be zwitterionic from studies on heats of ionisation (1) Raman spectra (2) and the phenomena of electrostriction (3). Amino-acids in their "neutral" state possess the formula: $\text{N}^+\text{H}_3\text{CHRCOO}^-$ and ionise according to the equations:



In dilute solution activities may be replaced by concentrations when the dissociation constants of the amino-acid as an acid and base are given by:

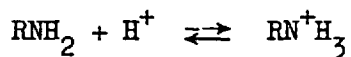
$$K_a = \frac{[\text{N}^+\text{H}_3\text{CHRCOO}^-] [\text{H}^+]}{[\text{NH}_3^+\text{CHRCOOH}]} \quad \text{and} \quad K_b = \frac{[\text{NH}_2\text{CHRCOO}^-] [\text{H}^+]}{[\text{N}^+\text{H}_3\text{CHRCOO}^-]}$$

When the concentrations of proton donor and acceptor are equal (i.e. at the isoelectric point)

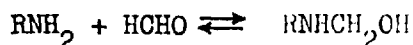
$$[\text{H}^+]^2 = K_a \cdot K_b ; \quad \text{or,} \quad \text{pH}_i = \frac{\text{p}K_a + \text{p}K_b}{2}$$

Proteins also display isoelectric points and by analogy with amino-acids might be assumed to be zwitter-ions at the isoelectric point. However this must be justified further since protein charge comes essentially only from the side chains, the amino-acid carboxyl

being used during polymeration. Also, many proteins are insoluble at the isoelectric point, thus appearing to have a low charge. This phenomenon might be explained by postulating mutual attraction between the opposite charges, effectively neutralising the molecule. Precipitation is then explained by inter-molecular electrostatic bonds. This interpretation gains some support from the phenomenon of "salting in", i.e. the prevention of precipitation of proteins at their isoelectric points by neutral salts. Neutral salts depress ionic double layers, with probable re-arrangement of the molecule such that only intra-molecular interactions are important. Such arguments are inconclusive but definite justification for the zwitterion form is obtained from the magnitude of the heat of neutralisation by strong acid or base (4) (5). A value of 10,000 cal per equiv is obtained when acid is added to the alkaline solution of protein at pH 9.0. This is very similar to the value obtained on neutralising organic bases and therefore corresponds to the equilibrium;



A further convincing indication is found in the comparison of the titration curves of proteins in the presence and absence of formaldehyde (6). This substance reacts with the ^{amine} ~~amide~~ group;



When proteins are titrated with NaOH in the presence of formaldehyde the inflection at about pH 9 ($\text{N}^+\text{H}_3 \rightarrow \text{NH}_2$) is shifted to lower pH, showing that the original buffering group was due to the amino-group and not the carboxyl ionisation.

Proteins are therefore safely assumed to have the zwitterionic structure near their iso-ionic points.

The complete structure of proteins is usually discussed at four levels; primary, secondary, tertiary and quaternary. The primary structure describes the amino-acid sequence of the protein molecules. The secondary structure describes the arrangement of each polypeptide chain, which is coiled as a result of bonds between elements of the polypeptide chains. The tertiary structure describes the arrangements in space of the coiled chains which lead to the characteristic protein molecule shapes. The quaternary structure refers to the organisation of macromolecular units in aggregates of proteins of high molecular weight, each macromolecular unit possessing its own primary, secondary and tertiary structure. Much of the direct evidence for the structure to be found in proteins (and their analogues, the synthetic polypeptides) has been derived from X-ray crystallography. Studies on single crystals can yield accurate molecular parameters for protein molecules, though up to the present time relatively few proteins

have been resolved in detail and these are of low molecular weight: e.g. myoglobin, Hemoglobin, lysozyme, ribonuclease, insulin. X-ray studies on solutions do not have such accuracy and an assumption about molecular shape has to be made, which is then matched with the actual behaviour of the real molecule. Values for shape and volume depend for the accuracy on how well the model duplicates behaviour over all conditions and because many studies are necessarily limited by their complication the models are often mathematical conveniences (7). It is not uncommon to infer structure in solution from single crystal studies. This approach is justified by indirect evidence that there appear to be no gross differences between the two states. Fortunately observations that absorption spectra and helix contents (as deduced from optical rotatory dispersion) are similar in both states are among the justifications for this approach. The most common justification is found in the fact that protein crystals contain large amounts of solvent, (typically 50% by volume), the crystal structure being unaffected even when the solvent is concentrated ammonium sulphate solution. This suggests that lattice forces do not cause much molecular distortion in the crystal. However, such studies have also been carried out only

on the well-characterised proteins of relatively low molecular weight and extension of this approach to higher molecular weight proteins may be of doubtful validity. In any case, minor differences in the structure of the two states (such as rotation of side chains or of the ends of the polypeptide chains) would remain undetected even at low molecular weight.

In the absence of other methods of greater accuracy (most other methods also require assumptions about molecular shape, density or hydration) this approach seems valid. Table II shows common techniques used in protein studies and the information obtained from them. The analysis is usually accomplished by the assumption that the molecular shape is an ellipsoid of revolution. Molecular parameters based on this model may then be calculated and compared for different proteins. The shape of molecules is not easily determined by X-ray diffraction because the molecules are so close together that it is difficult to decide where one ends and the next begins.

TABLE II

Techniques for studying proteins

<u>Technique</u>	<u>Information obtained</u>	<u>Purity required</u>
Diffusion	Molecular volume if shape and bound water are known	High
Sedimentation velocity	Molecular volume if shape and bound water are known	Intermediate

TABLE II continued

<u>Technique</u>	<u>Information obtained</u>	<u>Purity required</u>
Viscosity	Shape if bound water is known	High
Flow birefringence) Electric birefringence)	Shape if bound water is known	High
Light scattering	Anisotropy, molecular weight	Very High
X-ray scattering	Size and shape	Very High
X-ray crystal analysis	Size of molecules in crystalline form using an assumed shape	Very High
Electron microscopy	Size and shape of molecules after drying	High
Osmotic pressure	Molecular weight	Very High
Sedimentation equilibrium	Molecular weight	High

Most information concerning size and shape of protein molecules is obtained from radiation scattering experiments. Light scattering in the visible region is the most well known technique but since the limit of resolution between the various postulated shapes is $\lambda/20$ for the major protein molecule dimension (8) this technique only yields molecular weights for the smaller proteins. However, for large molecules such as the tobacco mosaic virus information

concerning shape is readily obtained. For the small proteins it is necessary to reduce the wavelength of the incident radiation and X-rays are used. The method has been reviewed recently by Kratky (9) and the ease with which radii of gyration of the scattering molecules may be obtained is clearly demonstrated. The actual shape is obtained by fitting the experimental curves to theoretically calculated curves. The molecular dimensions may then be determined from the radii of gyration. Extremely accurate measurements of the intensity of scattered X-rays over all observed angles of reflection are necessary and often difficulty is encountered in giving preference to one particular shape i.e. prism, ellipsoid etc. (10). Direct determination of molecular solvation may also be obtained by X-ray scattering. Adjustment of the solution density by inert substances so that it is the same as the protein core allows scattering by hydration sheaths to be studied. ^{It may be inferred from such studies} ~~Such studies infer~~ that internal hydration is usually present in proteins (11) especially the larger ones. The hydration sheath postulate stems from the idea that many of the hydratable groups of the protein molecules studied so far are found to be on the outside of the molecule by X-ray diffraction. It is also justified to some extent by viscosity studies on various proteins which show their behaviour to be closer to hard impenetrable spheres than random coils (Table III). This

may be envisaged if the major proportion of the hydrophobic side chains group together in the interior of the molecule to form a nucleus which is kept in solution by the hydrophilic groups surrounding it.

TABLE III

	<u>Intrinsic viscosity</u>
Rigid impenetrable sphere (theor.)	1.9
Random coil M.W. 50,000 (theor.)	47.0
B.S.A.	3.7
Pepsin	3.4
Ovalbumin	4.3

If the reported X-ray scattering dimensions are correct, the small differences shown in Table III represent quite large molecular hydrations (e.g., 50g water/g dry protein).

Other methods of determining molecular size e.g. ultra-centrifugation, have tended to give slightly different values but with the advent of modern methods of purification the latest results are in better agreement, especially when the results are compared after extrapolation to zero concentration.

Recapitulating, protein molecules in solution have the properties of solvated, globular masses. The solubility of the molecules is difficult to understand unless the hydrophobic portions of the

molecular backbone are shielded in the centre of the molecule with most of the charged and other hydrophilic groups essentially on the outside of the molecule. If the X-ray data on crystals are relevant to molecules in solution, this simple pictorial form is quite well justified for the proteins studied so far.

B. The Surface Chemistry of Proteins

There is extensive literature on spread monolayers of proteins at the air-water interface. Reviews are given by Bull (12), Cheesman and Davies (13) and Cumper and Alexander (14). Somewhat fewer studies are found for the oil-water interface because of the experimental difficulties and the reviews mentioned above cover much of the reported work, except for more recent surface rheological studies. The comparison between spread monolayers and adsorbed interfacial layers is discussed below.

The fact that foams and emulsions can be stabilised by proteins shows that they are surface-active substances and suggests that the hydrophilic/hydrophobic balance present in the native molecule is responsible in some way. From the considerations in the preceding section such a hypothesis for adsorption requires some degree of re-arrangement from the native form in order that non-polar side groups can be presented to the air or oil phase. This is manifest in the surface pressure-area (π -A) curves and the

Surface rheological parameter-area curves (η -A and ϵ -A) of spread surface layers; the area taken up by protein placed at air-water and oil-water interfaces at the point of minimum compressibility is greater than the cross-section area of the native molecule. This observation is not quite as clear as it might seem from the literature as most proteins are spread from solutions containing organic spreading solvent (15) or, alternatively, aqueous solutions are spread on substrates of high ionic strength (16). There is some evidence that the former method gives the surface properties of an organic solvent-protein complex (17) and results are not strictly comparable with those for protein. Spreading on salt solutions enhances unravelling by decreasing the solubility of protein in the bulk phase, thus making the surface region energetically favourable for the protein molecules and perhaps causing them to flatten and eventually re-arrange. Unfortunately many aqueous solutions of proteins have not been observed to spread reproducibly at interfaces (18). This would allow an unambiguous estimate of the degree of unravelling, on spreading proteins at interfaces. There are just a few results in the literature concerning ovalbumin spread from aqueous solution. These suggest that this protein does unravel extensively at the air-water interface since visco-elasticity is observed, and this decreases with increase of the ionic strength

of the substrate. However, at ionic strengths well below 0.1 the gelation area is still much larger than that which the molecules take up in their close packed state, indicating that some spreading occurs without solvent. It would not be justified to assume that these concepts are valid for other proteins that have not been examined; indeed, only fibrinogen has been observed to possess this property (19). The use of Trurnit's spreading technique shows promise of yielding unambiguous results for proteins spread from aqueous solutions but to date only two proteins have been studied, and these over restricted ranges of conditions; the two proteins are serum albumen (20) and serum globulin (21). The surface molecular area of BSA, calculated assuming adsorption in the native state is compared with actual results from monolayers in appendix I.

There is evidence that, even in solutions spread with the aid of solvents, such as isopropyl alcohol, proteins do not unravel completely at the air-water interface (22), (even those that do not contain internal disulphide bonds). The comparison of visco-elastic properties at the air-water and oil-water interface shows that the surface rheological properties at the latter interface becomes substantial at areas much larger than for the air-water interface. The observations are obtained from the behaviour of

synthetic polypeptides at the two interfaces. These may reasonably be expected to unravel to a high degree because of the absence of restricting covalent linkages. However, such substances unravel more at o/w than a/w interfaces. From the relatively few thorough studies on a small number of well characterised substances it appears that complete unravelling at the air-water interface is never encountered. Malcom (23) has presented results which show that there is some structural feature present even in monolayers spread with the aid of solvents. This work casts doubts on the idea that surface denaturation parallels heat denaturation. This may be investigated, in theory, if the thickness of surface layers can be measured. Thickness measurement has been attempted by various authors (24) (25) using interference techniques on protein layers removed from surfaces on conditioned plates. Spread monolayers yielded thicknesses about 10 Å and adsorbed layers about 15 to 20 Å. These may be regarded as minimum values because some drying must be done prior to experimental study, and also because a plate pushed through a stabilised interface increases the surface area, thus stretching the film. The latter effect may be readily demonstrated on the ^{Langmuir} ~~Langmuir~~-Adam balance (26). The classical work of Bull (27) on egg albumin indicated an initial 10 Å thick adsorbed layer. It is perhaps unfortunate that this protein seems to spread

to large areas per molecule without spreading solvent, and might not be a typical protein.

The increase in thickness with concentration was interpreted at one time in terms of multilayer structure, but the work of Joly (28) and the more recent work by Augenstein (29) (30), tend to support the postulate that adsorbed films consist of molecules of varying degrees of unfolding alongside one another. Justification for this idea is found in the observation that studies of surface pressure at constant area, for various surface concentrations, when converted to $\pi - A$ curves, are not the same as $\pi - A$ curves determined in the conventional manner. From such considerations surface films at the air-water interface might be expected to be thicker than that consisting of completely unravelled protein molecules. Again these studies have been carried out on relatively few proteins and care must be exercised in the use of the results. Surface layer thicknesses are often estimated from a knowledge of the area per unit amount of protein at interfaces. This requires knowledge of the value of surface density and as little is known about this parameter, especially in view of the fact that proteins can exist in several different forms at interfaces (see next section), ~~the~~ procedure must be regarded as capable of giving only comparable values and orders of magnitude. The use of "partial specific volume"

of the molecules in solution as though it represented the actual size of solute molecules also appears especially suspect.

Cumper and Alexander have discussed the mechanism of protein adsorption from solution (31). The adsorption is considered to occur in three main stages, which are (a) adsorption in the near globular form (b) surface denaturation of the adsorbed molecules (c) aggregation of the surface denatured protein into a coagulum largely devoid of surface activity because it is essentially insoluble. Depending on conditions, stages (b) and (c), or just stage (c) may be absent. Stage (c) has been reported absent under completely quiescent conditions (even with egg albumin) but McRitchie has reported visible solid coagulum formed from BSA on very concentrated salt solutions when monolayers are compressed to high surface pressures. From the few papers on this subject the most likely reason for surface coagulation is the high local pressure built up during mechanical agitation. Of the proteins studied, insulin is not known to coagulate (i.e. (c) absent) (32) while lysozyme is known not to be subject to surface ^{denaturation} ~~denature~~ at an appreciable rate at pH's below its iso-electric point; (b) and (c) absent.

Any coagulated material not broken away from the interface will exist alongside the unravelled, pressure re-arranged protein and

also the almost native molecules which have their unravelling opposed by the surface pressure. The surface thickness will thus be irregular when coagulation is present.

C. Protein Foams

A foam may be simply defined as a dispersion of gas in liquid, but several types of dispersion can be distinguished depending on the degree of "stabilisation". Foams are classified by using the observation that they drain to produce lamellae. Foams which yield lamellae persisting for long periods are classified as "persistent foams". Other types of foam may be called "transient foams". Viscous liquids may slow the rate of drainage between air bubbles to a rate such that bubbles can be injected to give a column of froth. However, observation of the bubbles shows that they burst on touching and thus there is no intrinsic stability. The term "foam" is, therefore, best reserved for systems of air dispersed in a liquid in which the air bubbles persist longer than in a pure liquid of the same viscosity (33), and produce thin lamellae between the bubbles after the stage of bubble contact is reached. Such foams are for all practical purposes produced only by solutions of surface-active agents.

When a foam is generated on top of a solution the solution trapped in the uppermost layers is subject to a hydrostatic, negative,

pressure (ρgh) and thus, providing the channels between the bubbles are not blocked, the liquid will drain away under gravity. When the liquid has been removed to such an extent that the air bubbles touch and then deform into flat lamellae a further suction force comes into play. The pressure in the Plateau borders (being constrained by curved surfaces) has a lower value than in the lamellae and this enhances drainage. Drainages by these mechanisms leads to the thinnest parts of the foam being at the edges of flat lamellae. These are thus the areas most prone to rupture. A study of the thickness and stability of free films (lamellae) will therefore yield information on the stability of foams. However, studies on lamella thickness do not yield much information on another major cause of foam instability, namely gas diffusion. De Vries (34) has derived an expression for the kinetics of foam destabilisation in the case where gas diffusion takes place without rupture of lamellae (for small times in viscous liquids) but no studies are reported for foams in the metastable state. Gas diffusion from the smaller bubbles to the larger ones gives rise to mechanically unstable packing, causing sudden rearrangement and rupture of lamellae. Studies on the rheology of lamellae (35) will yield some information on the resistance to sudden rearrangement; but the system is too highly complicated for simple study (41). Gas diffusion

is the prime cause of the difference in stability between foams and single lamellae (hours as compared to months or years for detergents).

Forces controlling the stability of lamellae

No foam may be strictly termed "stable" because of the large surface area contained within the system. However, a single lamella of persistent foam may be formed and kept indefinitely if protected from external disturbances. This corresponds to the thermodynamic metastable state. The ultimate properties of a foam thus depend on the influence of disturbances on individual lamellae. The formation of lamellae requires an increase in surface area. Thus one property necessary for foaming is surface elasticity. This property may arise in three ways:

(i) The Marangoni effect; an expanding surface on a solution of surfactant has a higher surface tension than the static value for the solution. Therefore disturbances are immediately met by a restoring force which tends to oppose either local thinning (increasing tension) or local thickening (decreasing tension).

Immediately after local extension of the film surfactant diffuses to the extended surface in order to re-establish equilibrium. If re-establishment of equilibrium is diffusion-controlled then less than a millisecond is needed for small surfactants (36) to cause "local" equilibrium. "Local" is used in the sense used by

Gibbs (37) meaning that equilibrium is reached in film elements having dimensions comparable with the film thickness, but not between neighbouring elements.

(ii) The Gibbs effect: There will be conditions when the amount of surfactant present in the film will be insufficient to restore the original concentration. Thus a persistent local rise in tension is caused. Gibbs defined an elasticity E as the ratio of the increased film tension ($2d$) to the fractional increment of film area ($d \ln A$) caused by the extension, i.e.

$$E = \frac{2 \, d\gamma}{d[\ln A]}$$

If a lamella of thickness ℓ is stretched by an area dA such that the thickness decreases by $d\ell$ and the tension increases by $d\gamma$

$$E = \frac{2 \, d\gamma}{d[\ln \ell]}$$

The Gibbs effect operates specifically in thin lamellae. Both effects are sometimes considered together and credit also given to the pioneer work of Plateau. The composite effect is sometimes known as the Plateau-Gibbs-Marangoni effect (38). This effect is responsible for the observations of optimum foaming conditions. Dilute solutions are unable to confer a large

restoring force because ($\gamma_{\text{dynamic}} - \gamma_{\text{static}}$) is small, and concentrated solutions relax the differential tension too rapidly for the restoring force to counteract the disturbance.

A study of the effect of minor components in surfactant solutions of Gibbs elasticity has been reported by van den Tempel (39). It has been shown that the addition of small amounts of highly surface active components to a detergent solution results in an increase in E under conditions where foam stability is also increased.

The fact that surfaces can support surface tension gradients allows the surfaces to be visualised as "elastic membranes" with the areas of high tension tending to contract. This results in movement of the surface itself. Since surface layers cannot slip over the substrate film liquid is taken with the film and thus brings bulk liquid into dangerously thin regions. This effect has been called "surface transport" by Ewers and Sutherland (40).

(iii) Surface shear elasticity; because of the visualisation of the surface of liquids as elastic membranes, investigators from the time of Plateau onwards have attempted to measure the surface rheological parameters of various systems. Many studies of surface shear viscosity are reported, but this parameter is probably not the significant one for foam stability. It has been pointed out

that viscosity is a dissipation of momentum (41) and cannot cause a restoring force. Viscosity will, however, have an effect on the rate of relaxation of stress but here the surface dilational viscosity is the necessary parameter (42). A method for determining this parameter has been described (43) and the theory verified (44). The results show that dilational viscosity is usually by no means Newtonian.

The full analysis of surface rheology requires four parameters, two viscosity coefficients and two elastic coefficients (45). This complicated problem has, however, been little studied. Protein surface layers have been studied and interpreted on the basis of models composed of classical Maxwell and Voigt shear elements and correlations have been found between lamella stability and the surface shear coefficients (46). This is probably an indication that dilational properties are simply related to shear properties for ~~irreversibly~~ ^{irreversibly} adsorbed films (47). The results show that both viscosity and elasticity can be correlated with film stability, thus being parallel with the observations on detergents (48). Measurement of the stabilising molecular thickness for polymeric detergents has led to suggestions of stability caused by molecular cohesion in the surface layers (49). Obviously when the surfaces are touching further thinning is impossible unless the surface layers intermingle and this is counteracted by forces of attraction between adjacent molecules

in surface layers. These films are, however, more easily formed in water-oil-water lamellae and by formally uncharged stabilising species. Such behaviour has not been previously observed in the air-water-air lamellae where electrostatic forces between double layers, and also between molecules in surface layers, are important (50). In the very thin ("second black") detergent lamellae there is still a little solvent between the adsorbed layers. There is, however, an indication that molecular interaction is important for the non-ionic detergents (51).

Forces controlling the thickness of foam lamellae

There are at least five forces controlling the thickness of foam lamellae; it is usual to express these in terms of pressure ($= \partial V / \partial r$ where V = potential, energy/cm² of a thin lamella, r = distance between the two air/water interfaces) ;

- (a) The Laplace suction pressure
- (b) The van der Waals pressure
- (c) The electrical double layer repulsion
between surfaces
- (d) The Born repulsion between adsorbed molecules
in opposing layers
- (e) Forces possibly arising from "solvation"
effects

The last two are very little understood.

(a) The lamella between two air bubbles in a column of foam will be under a hydrostatic pressure π_p given by

$$\pi_p = \rho gh \quad \text{where } \rho = \text{the density of the liquid}$$

$$g = \text{acceleration due to gravity}$$

$$h = \text{height of lamella above bulk solution}$$

The Laplace suction pressure will be constrained to this value such that;

$$\frac{-\gamma}{R_1} + \frac{-\gamma}{R_2} = -\rho gh \quad \text{where } \gamma = \text{surface tension}$$

$$R_1, R_2 = \text{radii of curvature of the two curved surfaces}$$

For bubbles constrained in a cylindrical support, and for small lamellae

$$= \frac{-2\gamma}{R} = -\rho gh$$

For other conditions Sheludko has shown the relation to be;

$$= -2R \frac{\gamma}{(R)^2 - (r)^2} = -\rho gh$$

where R = radius of curvature of the Laplace borders
 r = radius of the circular lamella

Pressures are regarded as positive if of repulsive nature.

(b) Molecules at liquid interfaces have non-symmetrical force fields around them. As a consequence there is a force towards the phase containing the highest concentration of surface component. When two interfaces approach one another such that the distance of separation is within the range of action of this force there is a net attraction between them. The attraction is numerically equal to that between ^{plates} ~~places~~ in a vacuum, as described by the equations of de Boer.

$$\pi_w = -A/6\pi h^3 \quad \text{where } A = \text{Hamaker-London constant for the material}$$

$$A = \pi^2 q^2 \lambda \quad \text{where } q = \text{number of particles per cm}^3 \text{ of material}$$

and λ is the interaction constant of two molecules of the medium and is defined by

$$U = -\lambda r^{-6}$$

(U being the free energy of two particles separated by a distance r). A review of this subject has been given recently by van Silfhout. (154) De Vries (52) pointed out that the attraction between two bodies of liquid in a vacuum is precisely the same as for the case of two cavities in a liquid and thus the attractive pressure is given by the formula of de Boer. An alternative derivation of the formula for the case of liquid lamellae has been given by Kitchener (53), and leads to the de Boer formula. The constant A must be modified for two bodies of the same kind O embedded in a medium l . Hamaker and de Boer (54) give;

$$A^* = \pi^2 (q_1^2 \lambda_{11} + q_o^2 \lambda_{oo} - 2q_1 q_o \lambda_{ol})$$

$$\text{or } A^* = A_{11} + A_{oo} - 2A_{ol}$$

For the case of liquid films in air A_{oo} and A_{ol} may be negligible compared with A_{11} .

There are, however, two complications to the above considerations.

The first occurs at large thicknesses due to retardation, i.e. accounting for the finite velocity of energy transmission. Casimir and Polder (55) have corrected the London equation from dependence on r^{-6} to r^{-7} . This results in a h^{-4} law of force for parallel walls. Studies on free films have been reported by Shelduko (56) and Lyklema and Mysels (57), and the theory discussed also by Kitchener and Read (58). The second complication is the actual structure of stabilised films. At thicknesses less than a few hundred Angstroms they cannot be regarded as homogeneous because of the presence of the stabilising species in the surface layers. Hence complications ⁱⁿ estimating A appear.

(c) The surfaces of free films generally possess electrical double layers similar to those between colloidal particles in solution. The source may be either charged stabilising species or charges adsorbed by nominally uncharged species. Such charges will attract their layers of counterions and the double layer is thus set up. The equations for the repulsion pressure are usually calculated for constant surface potential i.e. based on the assumption that there is thermodynamic equilibrium everywhere in the double layer. Verwey and Overbeek (59) have indicated that in certain cases energy barriers may occur between surface and bulk for ions and then surface charge is the parameter independent

of distance. These cases were, however, considered exceptional and emphasis placed on the constant potential model, though the above authors conclude that the order of magnitude of the repulsion would not be greatly affected by the assumption of either (50) approximation. Sheludko and Exerowa have shown that Π_e can be calculated with sufficient accuracy from the approximate formula;

$$\Pi_e = 64nkT^2 \exp(-\kappa h)$$

where n = concentration of 1 : 1 electrolyte
in molecules cm^{-3}

κ is given by

$$\kappa = \left(\frac{8\pi z^2 e^2 n}{\epsilon kT} \right)$$

$$\gamma = \frac{\exp(e\psi_0/2kT) - 1}{\exp(e\psi_0/2kT) + 1}$$

e = elementary charge

ψ_0 = potential at boundary walls

k = Boltzmann constant

T = Temperature

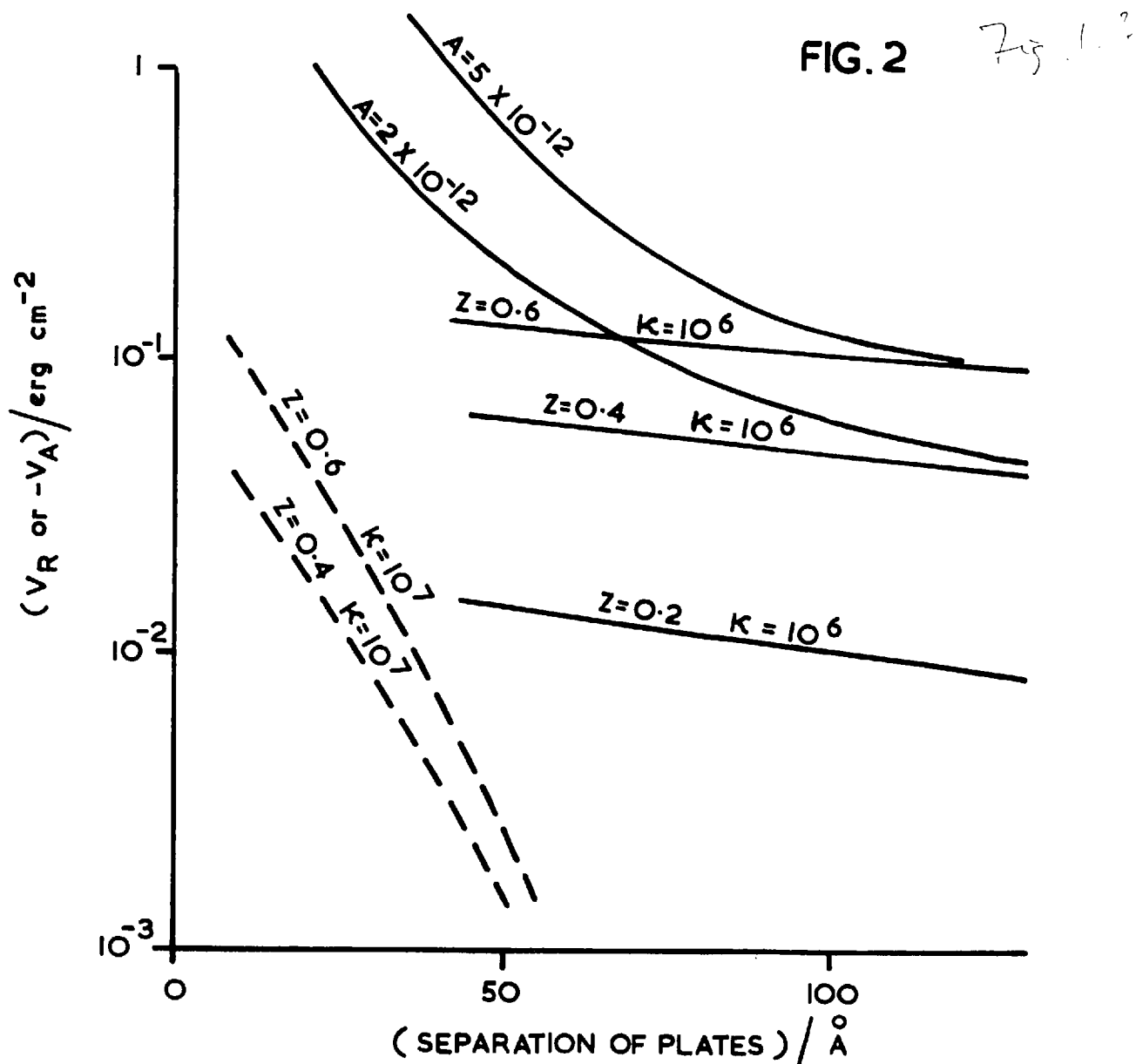
ϵ = dielectric constant

z = valency of counterions

The summation of these forces leads to the classical interaction curves of colloid chemistry. Typical potential energy-distance curves are shown in fig 2. Pressure distance curves may be obtained from the slopes of these curves since $\Pi = \partial v / \partial x$. Fig 2 shows specific curves for low values of ψ_0 at separations up to 100 Å or so, for different ionic strengths.

POTENTIAL ENERGY - THICKNESS CURVES

FOR FLAT PLATES



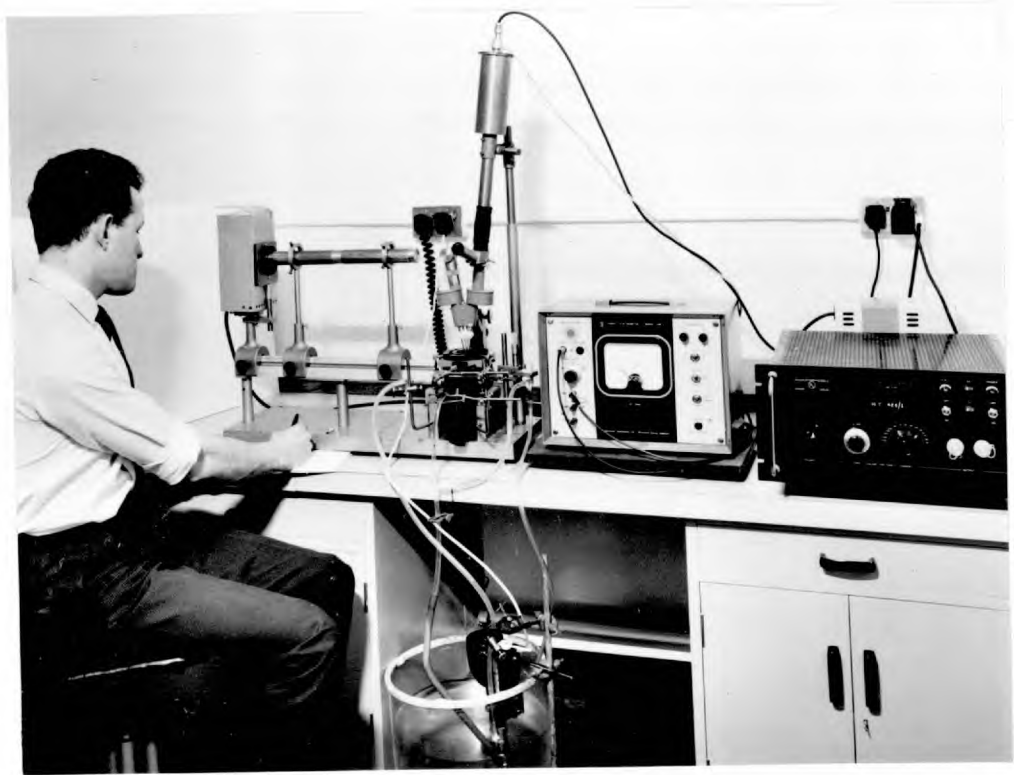
2. Experimental method

(a) Choice of method

Individual lamellae may be supported in the vertical or horizontal plane. In either case the greatest stability is found for small films. Preliminary studies on vertical frames similar to those used by Mysels (60) showed that protein solutions yielded films which would thin to the black state, but these burst before an appreciable area of black film was formed. Often the black film appeared only at one corner of the frame and did not spread before rupture occurred. Therefore, the horizontal method has been adopted.

Of the two forms of apparatus described in the literature, that of Sheludko (61) appeared most convenient for miniaturisation, and has been used in preference to the apparatus described by Derjaguin (62).

The Sheludko method consists of applying capillary suction to two hemispherical menisci which slowly removes the interlamella liquid causing the surfaces to approach one another. In his apparatus Sheludko uses the surface tension and the radius of the tube to control the pressure ($P = 2\gamma/r$). Since proteins do not yield a constant value of γ for quite long times, the method has not been utilised intact but has been modified as described below.



The Apparatus
(With light shielding removed for demonstration)

Fig 2.

APPARATUS FOR MEASURING THE REFLECTIVITY OF THIN FILMS

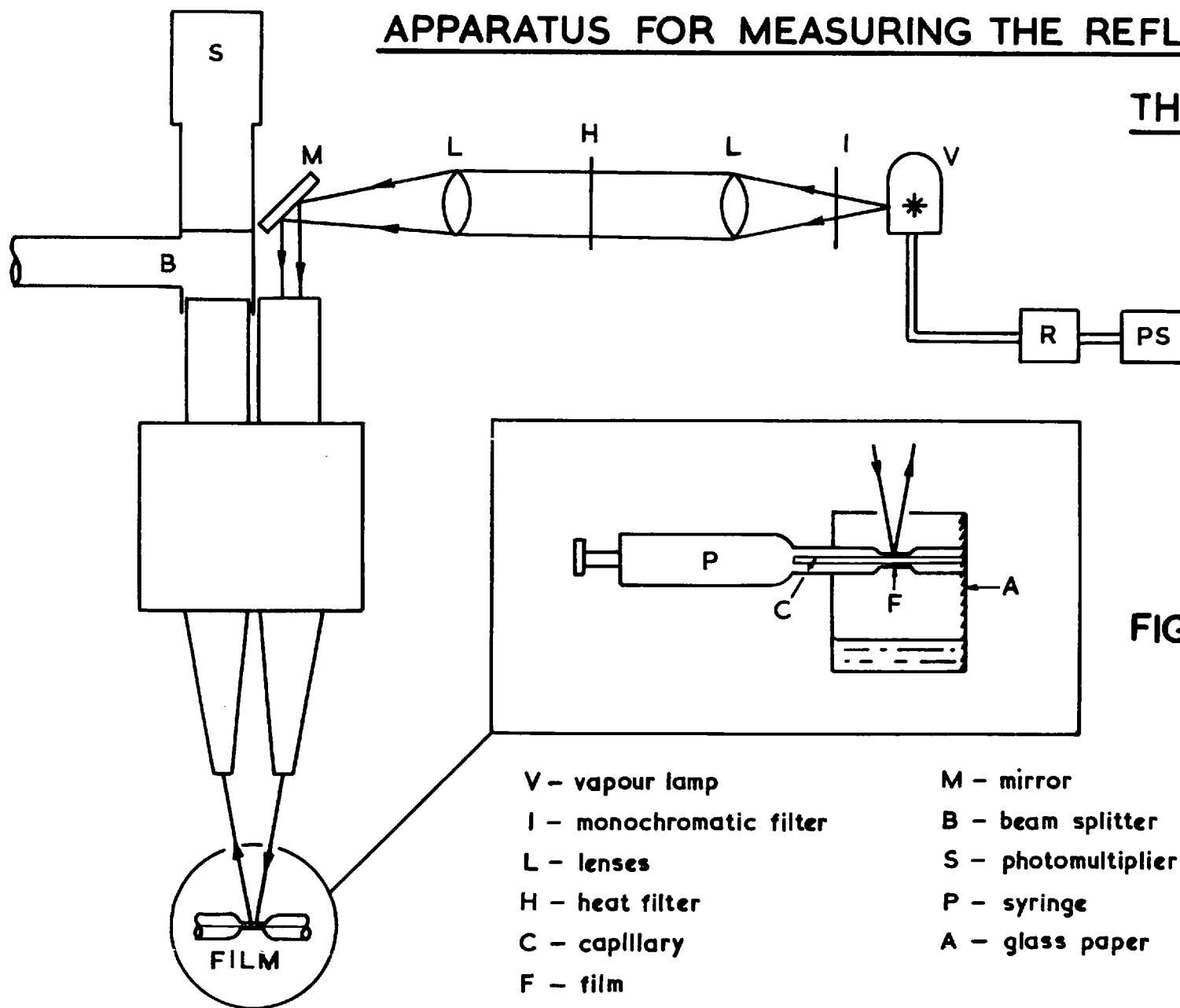
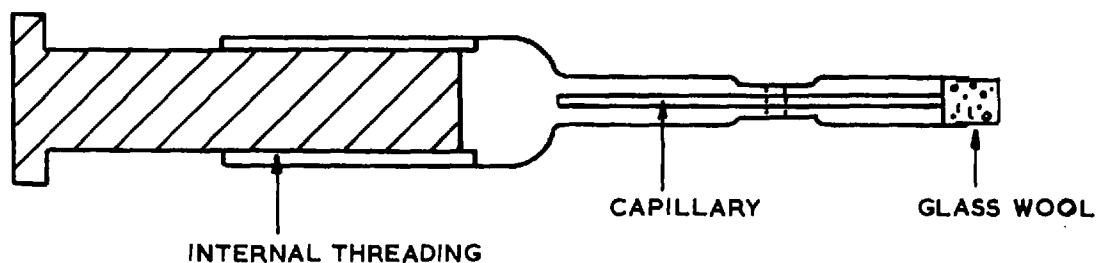


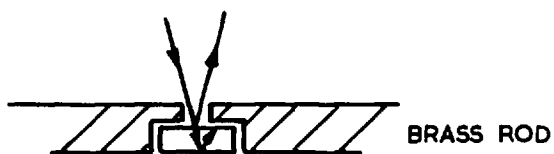
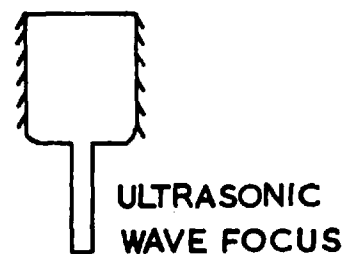
FIG.3

- | | |
|--------------------------|---------------------|
| V - vapour lamp | M - mirror |
| I - monochromatic filter | B - beam splitter |
| L - lenses | S - photomultiplier |
| H - heat filter | P - syringe |
| C - capillary | A - glass paper |
| F - film | |

FIG.4

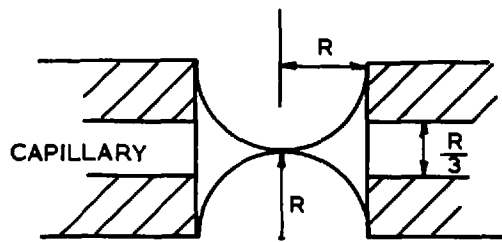


FILM SUPPORT FILLING PLUNGER AND
FINE VOLUME ADJUSTER.



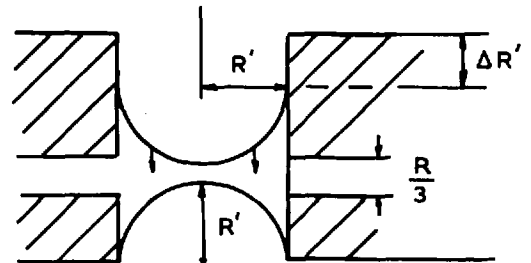
SUPPORT FOR QUARTZ PLATE

FIG.5

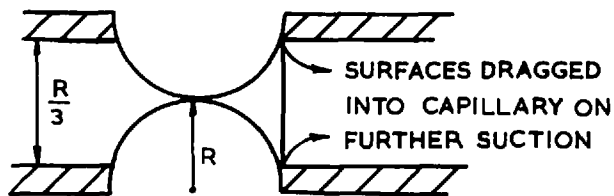


IDEAL SYMMETRICAL FILM
SUPPORT DESIGN

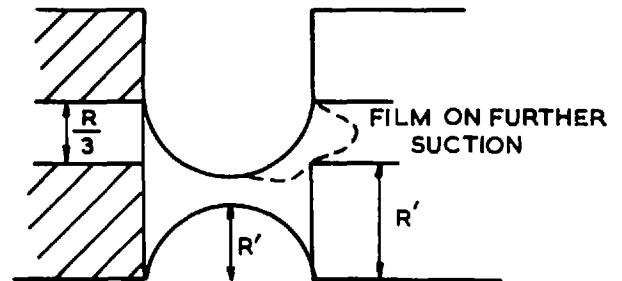
$$R > R'$$



ACTUAL FILM SUPPORT
DESIGN FOR SMALL R' (i.e. 1mm)
 $\Delta R'$ ALLOWED FOR STRENGTH



CAPILLARY TOO LARGE.



CAPILLARY $< R/3$ BUT TOO
HIGH UP ORIFICE.

The design of various film supports

The Film Support

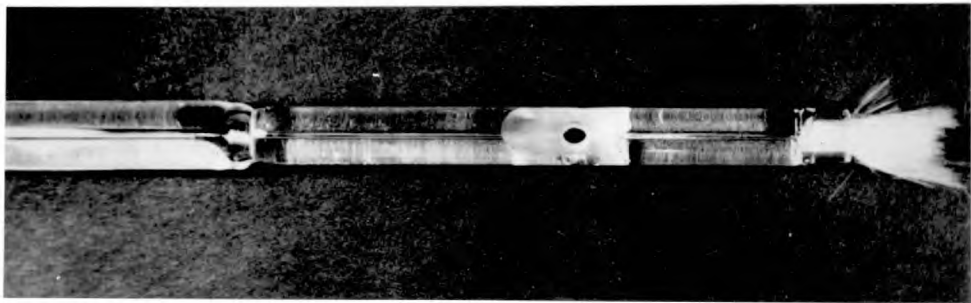


Figure 5(a)

The measurement of film thickness is best carried out by means of a light sensitive device to avoid eye strain and operator bias, and here again the method of Sheludko has been closely followed. The one major difference is the choice of a small angle of incident ($7\frac{1}{2}^{\circ}$ to the normal) for the incident light, as described by Duyvis (63). This ensures maximum collection of reflected light, and ensures the absence of stray light from the transmitted beam.

(b) Apparatus

The apparatus is shown in figs 3,4,5. The lamella support was a cylindrical orifice which had been drilled through a piece of Pyrex capillary tube using a "Mullard" ultrasonic drill. This was drilled with an ultrasonic wave focus made by turning down a thread which fitted the drill (shown inset fig 4). The diameter of the orifice (2R) could be made as small as 1mm with little difficulty. Smaller orifices were difficult to prepare because of "whip" in the thin focusing device. The top and the bottom of the orifice were ground flat with a diamond wheel such that the overall height was of the order of twice the radius, the controlling consideration being that a beam of light at $7\frac{1}{2}^{\circ}$ to the axis of the orifice did not strike the sides of the orifice. Experiments on cells of various sizes showed that the radius of

the capillary had to be no larger than $R/3$ to avoid the surfaces of the lamellae being sucked into it by capillarity during the thinning process. For very small values of R it was found necessary to make the orifice heights larger than $2R$ for strength, and the tube was ground so that the capillary was at a distance approximately R from the bottom of the orifice, and at a distance greater than R from the top. This prevented the top film interface being sucked into the capillary. (This appeared to be caused by the weight of solutions in the orifice causing preferential movement of the top interface unless the cell was exactly symmetrical with height $2R$ and radius R). Since the drilling was controlled by eye, absolutely symmetrical cells were difficult to obtain and grinding by trial and error had to be employed. Attempts to angle the sides of the orifice to produce symmetrical movement were not successful. The various forms of the cells are shown in fig 5.

The film support was mounted in a box blacked out both internally and externally with "matt black" paint. The support entered via two internal rubber "O" rings and the plunger on the outside rested on a metal frame. Knurled screws beneath the supporting sheath allowed for adjustment of elevation of both the film support and the piece of optical quartz plate which was

used as the optical standard. The latter was attached to the equivalent of the glass film support, made in brass, such that the reflection from the bottom quartz surface fell on the underside of the brass (fig 4). In this way only one reflection was "seen" by the photomultiplier. Suction was applied to the film by means of capillary contact, through the tube via a glass wool "wick" to the bulk liquid in the body of the box. This allowed variation of the hydrostatic pressure (at the expense of symmetrical shape of the surfaces) by adjusting the level of the bulk liquid. The inside of the box consisted of a central light trap and an outer reservoir for water, the latter being used for applying the hydrostatic pressure and also for temperature control to $\pm 1^{\circ}\text{C}$. The level of water in the box was controlled by a constant head device adjusted by means of a micrometer (calibrated to 0.01mm). In order to prevent stray light entering the box all tubing was painted black. The whole box was supported on the stage of a binocular microscope with three adjusting screws for positioning beneath the light beam. The top of the box was closed by a bellows made from heavyweight blackout cloth which was attached to the rim of the box and the binocular objective. This allowed for focusing adjustments of the microscope. In order to ensure saturation of the air inside the box the cloth was kept

saturated by a trickle of water from the reservoir.

The light beam was focused down to a small spot through one side of a binocular microscope with pin hole diaphragms in the eyepiece at the top of the objective. The symmetrical form of the objective ($7\frac{1}{2}^{\circ}$ to vertical) allowed the light reflected from a surface in the horizontal plane to be collected by the other side of the microscope and then enlarged in the diameter, by removing the top eyepiece, before falling into an 11-stage photomultiplier. By mounting the photomultiplier 25 cm from the top of the microscope a spot approximately 4 mm in diameter was obtained, which then illuminated a 10 mm diameter photocathode. This precaution was taken to avoid errors that might have arisen with a very small image falling on different areas of the cathode which were of differing sensitivity.

Initial experiments were carried out with a mercury vapour lamp run from a stabilised D.C. supply. This procedure yielded a stability in intensity of $\pm 5\%$. However, the position of the arc was prone to change especially for lamps used for more than 20 hours, yielding a different intensity also stable to $\pm 5\%$. It was, however, relatively easy to detect the shift in arc position by eye as well as using the photomultiplier. The use of lamps with more than 20 hours running time necessitated measurement

of incident intensity during the experiment and after it. Mercury vapour sources necessitated the use of two lenses to collimate the beam before entering the microscope. Later experiments were carried out with a helium/neon gas laser which required only one lens to focus the beam of light into the microscope since the laser mirrors with focal lengths of 1 metre produced a slowly diverging beam of light, which at the operating distance of one metre was smaller in area than the lenses used in the study, and collimation was thus unnecessary. The laser produced light at two main wavelengths $6,328 \overset{\circ}{\text{\AA}}$ and $11,523 \overset{\circ}{\text{\AA}}$, the latter being removed by two glass filters. The fact that all of the laser light could be made to enter the microscope conferred a very high incident intensity allowing the photomultiplier to be run at lower voltages than for the mercury arc, despite the sensitivity lowering by a factor of ten caused by the increase in wavelength from $5,470 \overset{\circ}{\text{\AA}}$ to $6,328 \overset{\circ}{\text{\AA}}$. Lower voltages reduced the background signal of the photomultiplier to much less than the signals obtained from free, black, protein films. The intensities of reflection from films were compared with the reflection from a piece of polished silica glass, using neutral density filters to reduce the intensities from the silica to the same order as those from the films. A direct record of the thinning processes was obtained

PHOTOMULTIPLIER CIRCUIT

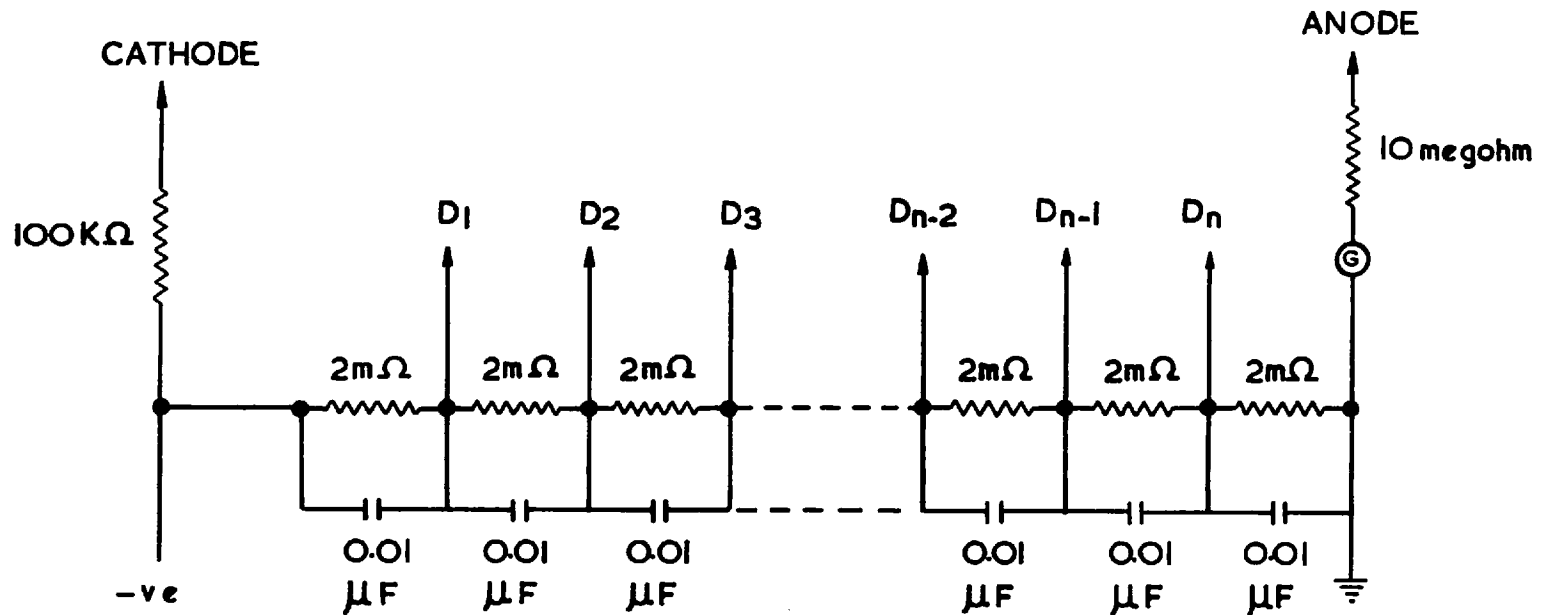


FIG. 6

by dropping the anode currents across high stability resistances and measuring the voltages obtained on a recording galvanometer. The photomultiplier circuit diagram is shown in fig 6 .

(c) Characterisation of supporting orifice.

For any one film support predictions may be made concerning the shape of the film surfaces and the suction pressures acting on them. The surfaces are not exactly hemispherical, because of gravity, and the ratio of the radius of curvature (b) at the lowest point of the meniscus are greater than the radius of the supporting tube (r). (i.e. the surface is flattened at the bottom). The ratio r/b is given by the tables of Bashforth and Adams (64) or of Sugden (65). A reproduction of a composite table is given by Adam (66) together with the method of successive approximation for b (which is, for complete wetting of the tube).

- (i) approximate the value of a (the capillary constant) from the formula $2\gamma/g(D-d) = a^2 = rh$

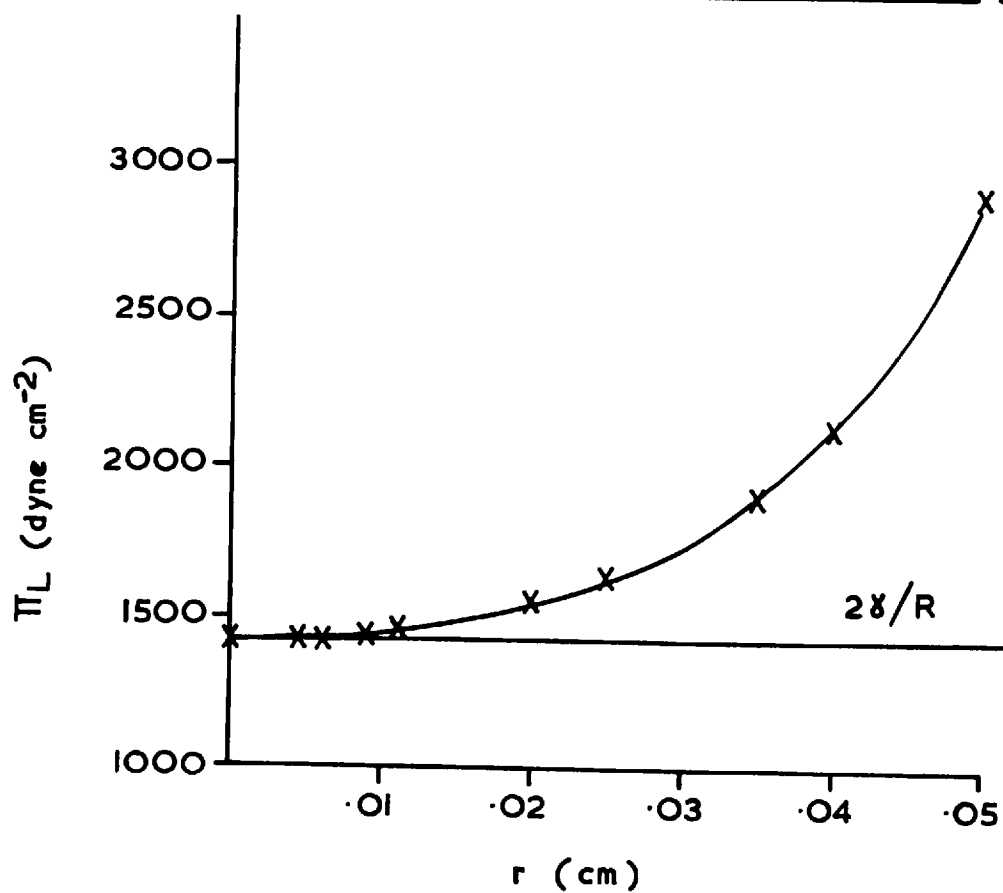
where γ = surface tension
 g = acceleration due to gravity
 D = density of dense phase
 d = density of light phase
 h = height of capillary rise

- (ii) calculate r/a and look up r/b in the tables
 (iii) calculate b and recompute a from $a^2 = bh$ (h determined by experiment).

FIG.7

PLOT OF $2\gamma R/(R^2-r^2)$

COMPARED WITH $2\gamma/R$



(iv) successively approximate to constant b .

Table IV shows results for typical values of r and γ
[calculated from steps (i) and (ii)]

TABLE IV

(dyne cm^{-1})	$r(\text{cm})$	$b(\text{cm})$
50	0.07	0.0711
30	0.07	0.0719
50	0.05	0.0504
30	0.05	0.0507

Thus the surfaces are essentially hemispherical.

The Laplace suction pressure on two surfaces constrained by
a tube of radius R is given by Sheludko (67) as,

$$P = 2\gamma R / (R^2 - r^2)$$

where r is the radius of the circular plano parallel lamella formed
on contact. Fig.7 shows the results for typical apparatus
dimensions. $R = 0.007 \text{ cm}$, $\gamma = 50 \text{ dyne cm}^{-1}$.

(d) The water still

All solutions have been prepared with very high purity water
obtained by stripping drinking water of both organic and inorganic
impurities.

The preparation of water without surfactant impurities is
notoriously laborious. Treatment of tap water with ion exchange
resin (68) activated charcoal (69) or simple distillation all have
limitations and combinations of the various methods must be used.

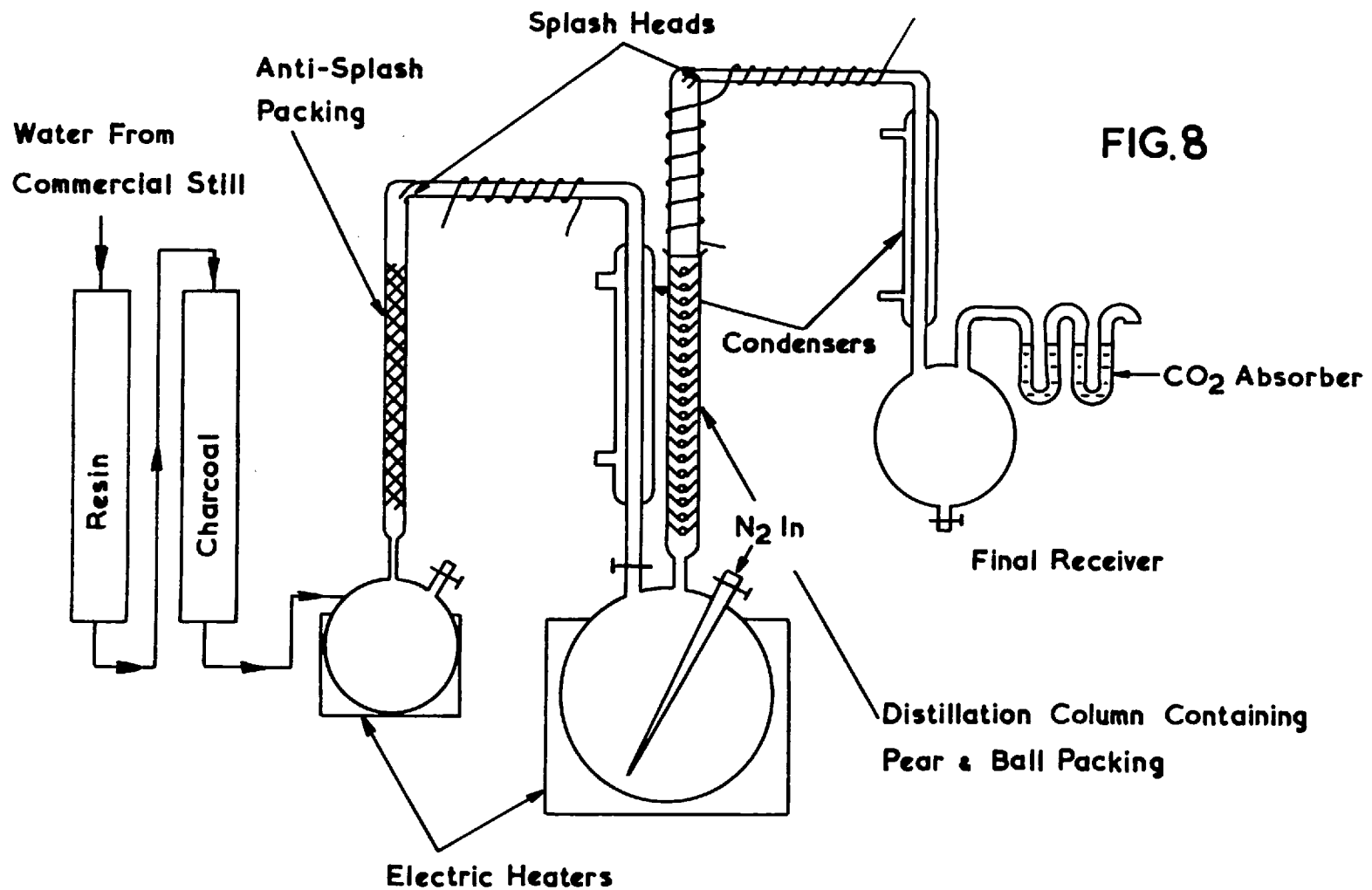
The water was given four stages of purification;

- (i) Initial distillation
- (ii) De-ionisation
- (iii) Treatment with activated charcoal
- (iv) Final distillation

Stage (i) was executed using a commercial water still and was only necessary because of the hardness of London water. This initial stage reduced the number of regenerations of the mixed bed resin used in stage (ii). The resin chosen was a mixture of strong acid and strong basic resins, analytical grade.

Stage (iii) removed any organic substances present after de-ionisation (70). Even after excessive leaching of the resins, water passed through them still had a residual foaming capacity which necessitated treatment by charcoal. It was found necessary to leach the charcoal with both acid and alkali to remove impurities and to fire at 800°C under nitrogen before use. Such treatment produced an adsorbent which almost completely removed the residual foamability of the water after passage through the resin column. The charcoal did, however, introduce some inorganic material, as shown by an increase in conductivity. To remove residual inorganic and organic material a double state still was built in Pyrex glass and silica. This is shown in fig 8.

Water Purification System



Ion Creepage up Fractionating Column (7l)
demonstrated by heating an area to 110°C



Figure 8a

The first stage consisted of a simple distillation up a column packed with Raschig rings to prevent splash. The top of the column was heated with resistance wire to prevent ion creepage along the column surfaces (71). This first stage effectively removed inorganic ions but a second was required for removal of organic matter.

A fractionating column was built containing "pear and ball" inserts to provide a "scrubbing" action on the vapours. In order to keep water in the column, a close fit was required between the pears and the supporting tube. After a while, however, this led to the column filling with water which splashed onto the heated part of the apparatus causing periodic cracking of the glass. This was overcome by making the top part of the column of silica tube and heating it to just above 100°C . A piece of silica tubing yielding low contamination was found by trial and error. The vapours were condensed in a Pyrex glass condenser and collected in a flask in a stream of nitrogen. Attempts to use a silica condenser failed because of electrolytic surfactant contamination which was present even after leaching for three months. The boiler for the last stage was of five litre capacity and the first litre was used to steam the apparatus^a each morning. The middle two litres were then collected for experimentation. Using this technique,

water of conductivity $0.2 \times 10^{-6} \text{ ohm}^{-1} \text{ cm}^{-1}$ was easily obtained which showed no detectable foam stability on shaking or on releasing a bubble beneath a water-nitrogen interface.

3. Theory of method of calculating thickness from reflectivity.

(i) The simplest model of a free film is a single layer confined by two surfaces of equal and opposite reflection coefficients. The formula for the ratio of reflected intensity (I) to incident intensity (I_0) was given by Rayleigh (72) as,

$$I/I_0 = \frac{4(n-n_0)^2/(n+n_0)^2(2\pi nd/\lambda)\cos\phi}{1-2\left[(n-n_0)^2/(n+n_0)^2\cos(4\pi nd/\lambda)\cos\phi + (n-n_0)/(n+n_0)\right]^4}$$

where n = refractive index of film
 n_0 = refractive index of surrounding medium
 d = thickness of film
 λ = wavelength of incident light
 ϕ = angle of refraction of film

It is usual to write $r = (n-n_0)/(n+n_0)$

$$\beta = (2\pi nd/\lambda)\cos\phi$$

For dilute aqueous of surfactants, the quadratic term in r may be neglected provided n_0 is close to the value of n . For small values of r the Rayleigh equation may be approximated to

$$I/I_0 = 4r^2\sin^2\beta \quad \dots\dots(2)$$

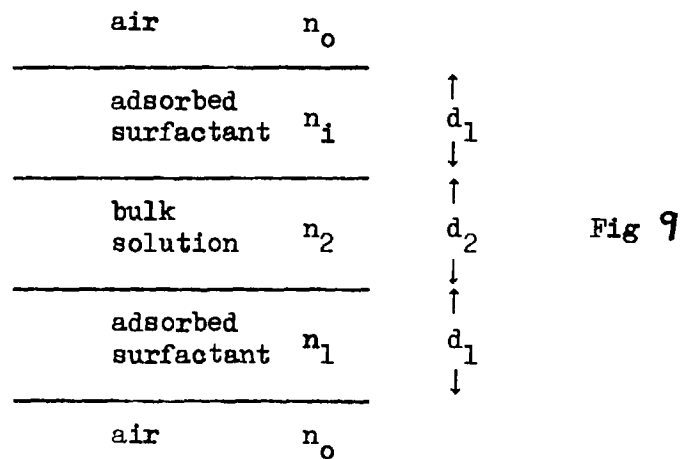
This is more justified, however, for films between oil and water than for air and water. For the latter case the Rayleigh formula

may be arranged to

$$I/I_o = \frac{4r^2 \sin^2 \beta}{(1-r^2 \cos 2\beta)^2} \quad \dots\dots (3)$$

This equation need only be used when there is no information in interfacial thicknesses etc. The results indicate that many protein films get no thinner than about 100 Å and have refractive indices around 1.5. For such values of film parameters I/I_o changes with distance such that a 4% error incurred through ignoring the denominator leads to about a 2% error in calculation of d . Because films are, in fact, sandwich structures, it is doubtful whether accuracy greater than that given by equation (2) is worthwhile.

(ii) The "sandwich model" for free films (first used by Duyvis) is shown in fig 9 .



In this case (41) $I/I_0 = a^2/(b^2+c^2)$ (4)

where $a = 2 r_1 \sin(2\beta_1+\beta_2) - r_1 r_2^2 \sin(2\beta_1-\beta_2) + (1+r_1^2)r_2 \sin \beta_2$

$b = (1-r_1^2) \cos(2\beta_1+\beta_2) - r_2^2 \cos(2\beta_1-\beta_2)$

$c = (1+r_1^2) \sin 2\beta_1+\beta_2) - r_2^2 \sin(2\beta_1-\beta_2) + 4r_1 r_2 \sin \beta_2$

$$r_2 = \frac{n_2-n_1}{n_2+n_1} \quad r_1 = \frac{n_1-n_0}{n_1+n_0} \quad \beta_1 = 2\pi n_1 d_1/\lambda \quad \beta_2 = 2\pi n_2 d_2/\lambda$$

Graphs of I/I_0 against d may thus be prepared if the various parameters are accessible.

Various methods have been reported for using the simple Rayleigh formula, which after application of a correction factor yields the true thickness (73). However, the sandwich formulae can be solved simply on a computer and this method has been adopted here. Undoubtedly the films are more complicated than the three layer approximation, but little is gained from such considerations since uncertainty is present, even in the simplest models. For instance, the refractive index of surface layers may be obtained from Brewster angle measurements but since there is probably still some aqueous core left, even in the thinnest state, the values are probably not highly accurate unless analysed according to the method of Corkill et al (74). Even the results from this method have been questioned because the molecular

extinction coefficient for water in very thin films is in dispute (75). Even when agreement is reached on the parameters needed for a full analysis the interpretation of measurements on aqueous core thicknesses is still uncertain because of the hydrophilic nature of parts of the stabilising molecules. (This uncertainty is particularly great with proteins). The three layer model will, however, yield more reliable values than ~~for~~ the homogeneous approximation.

Calibration curves of I/I_0 against thickness ($2d_1 + d_2$) may be drawn if the parameters n_1 , n_2 , d_1 , d_2 , λ , can be determined. λ and n_2 may be determined directly but n_1 and d_1 present difficulties. The latter parameters could be determined directly by measuring I/I_0 at two different values of λ and using the Rayleigh formula if the films thinned down to a true bimolecular layer. There is, however, no evidence that protein films thin to this state, and, therefore, at least one parameter must be estimated. n_1 may be determined from the Brewster angle of the film, but since proteins do not form stable films of large area it is impossible to shine a beam of light onto them at an angle of incidence of around 50° without striking the film supports. Thus for this study both n_1 and d_1 have been estimated.

(a) The refractive index of surface layers

The refractive indices of solid proteins are usually in the region of 1.6. This has been estimated from the Lorentz (76) equation and also measured directly on prepared plates (77). The refractive index of the surface layer, therefore, must lie between 1.33 (i.e. that of pure water) and 1.6. The actual value will depend on the proportion of water in the surface layers, and this will consist of both "bound" and "free" water. Since the layers are formed in the presence of air saturated with water vapour, it is to be expected that the refractive index will be greater than that of the most concentrated solution reported in the literature and less than that of protein crystals. Barer and Tkaczyk (78) reported the values of the refractive index of serum albumin solutions concentrated by pressure dialysis to concentration up to 50 g protein per 100 g water. In this case the refractive index lies between 1.42 and 1.6. A smaller range may be obtained by assuming that the upper limit is that of protein containing the classical 20 g water per 100 g dry protein. Linear interpolation between these limits yields refractive indices for reported values of the hydration of native serum albumin (79) of around 1.5.

Another approach can be used to check the feasibility of this approach. It is well known that the empirical formula

$$n = \phi_1 n_1 + \phi_2 n_2 \quad \begin{array}{l} \phi = \text{vol. fraction of 1} \\ n_1 = \text{refractive index of 1} \end{array}$$

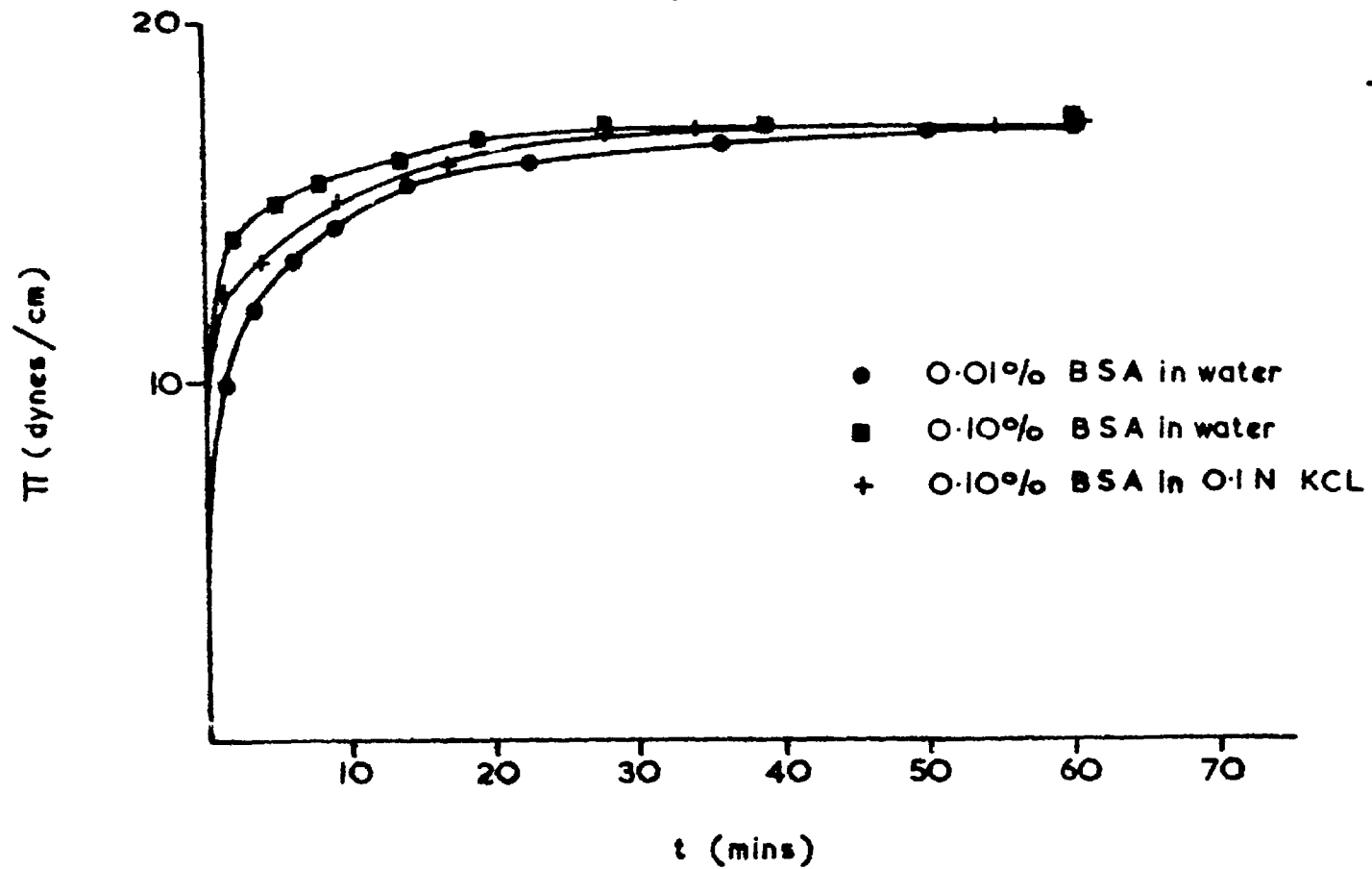
is surprisingly accurate. Substitution of reasonable values of the parameters yields values of n between 1.40 and 1.55. This method is however artificial since it uses apparent volumes in the calculation of ϕ , but it does provide a crude check.

(b) Thickness of surface layers

The thickness of monolayers of various proteins has been estimated to be about $10 \text{ \AA}^0$. The classical work of Bull on egg albumin showed that the apparent thickness of a "completed" adsorbed layer increased with bulk concentration. However, the lowest speed of revolution of his drum only allowed 4.1 secs. for adsorption and the value $10.6 \text{ \AA}^0$ has been extrapolated from this time. Adsorption of proteins progresses over a period of hours (fig 10) and thus any thickness value can only be approximate. The values obtained on prepared plates must also be accepted with caution. Inevitably some drying of the plates must be carried out and this could lead to variation in molecular volume, and hence thickness. This is probably the reason for the irreproducible

Pressure - time curves for B S A

Fig. 10



values of the refractive index found by many authors using this method (80). A method of estimating adsorbed surface thickness that is available only for the oil-water interface is adsorption measurements on emulsions. If the surface area per unit amount of protein can be obtained then combination of this result and the value of the surface density will yield the thickness. Unfortunately the value of the latter parameter is unknown, and it is usual to use a density estimated from the partial specific volume of the molecules in solution ($\rho = 1/\bar{v}$). Approximate values obtained by Cockbain (81) and Biswas (82) again were in the region of $10 \text{ \AA}$ for concentrations around 0.01% .

Because of the uncertainty in the values of the necessary parameters required for calculating thickness of films from reflectivity values calibration curves have been plotted to yield maximum and minimum values of d_3 for given values of I/I_0 , using acceptable extreme values of the parameters n_1 and d_1 . These values have been chosen at 10 and $20 \text{ \AA}$ for d_1 and 1.44 and 1.52 for n_1 . The curve for $d_1 = 15.3 \text{ \AA}$ and $n_1 = 1.47$ falls approximately half way between the extremes and has been chosen to analyse the results for bovine serum albumin (BSA). Providing the extreme values have been correctly chosen the uncertainty involved has a maximum value of $\pm 7 \text{ \AA}$ for $I/I_0 = 0.1\%$ i.e. $d_3 \approx 100 \text{ \AA}$. Fig. 11

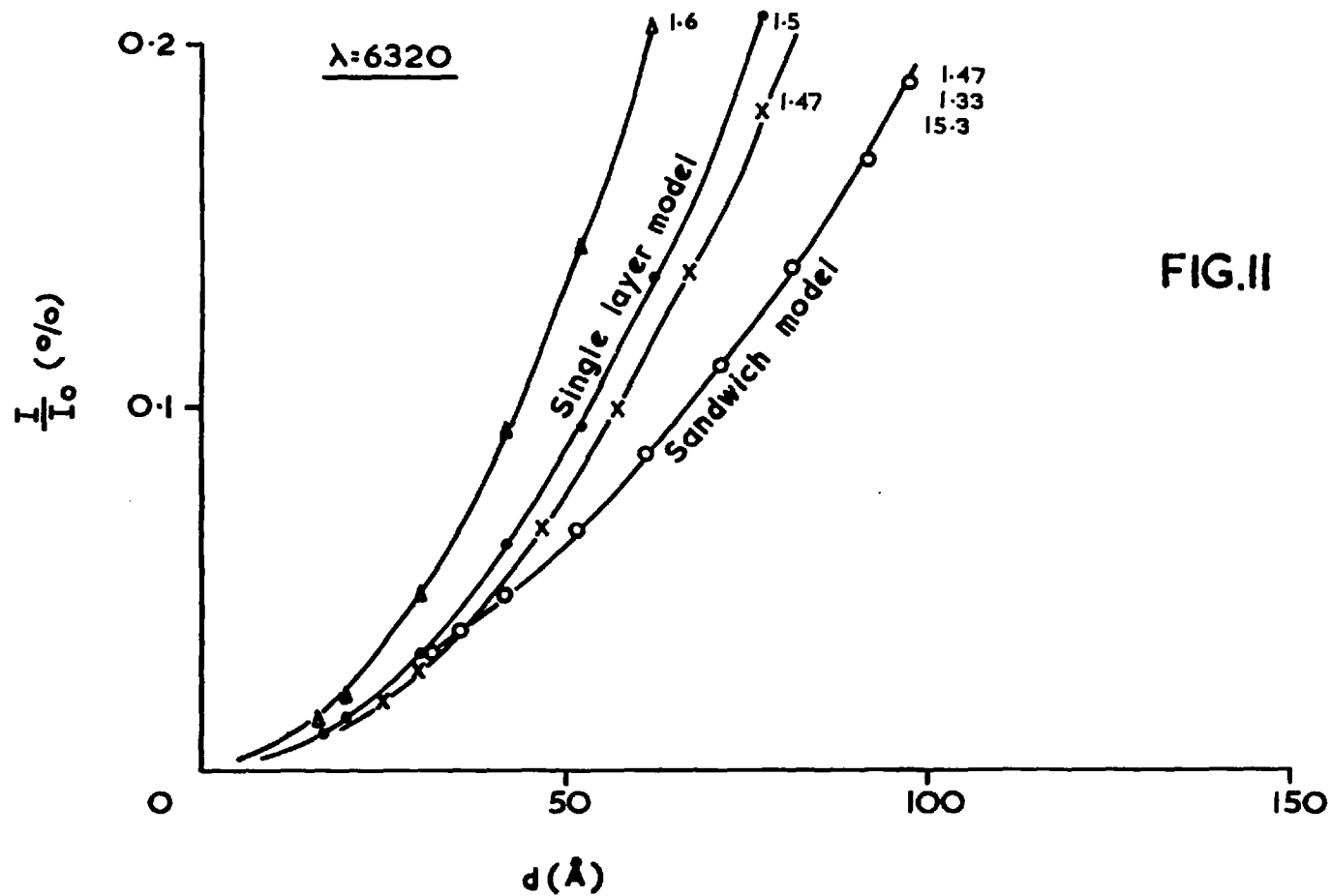


FIG.II

Thickness-reflectivity curves for various values of surface layer refractive index.

4. Choice of proteins

Of the proteins commercially available in an acceptable state of purity for the present research, the blood plasma proteins are probably the best characterised with regard to their bulk solution properties. Of these, the serum albumin fraction of bovine blood (BSA) is often used as a typical, well characterised, protein and it is conveniently soluble over the whole pH range. Many studies on this protein have indicated high degrees of purity for commercial samples. Well dried samples, stored at 0°C for short periods of time, contain between 2 and 5% of a high molecular weight component which is probably the BSA dimer. Although the primary structure of BSA has not been elucidated, much work has been reported on the size and shape of the molecule in solution and other necessary parameters are accessible, subject to plausible assumptions and interpolations. BSA has also been used for several recent surface chemical studies allowing estimates of parameters such as surface thickness to be obtained.

Other proteins such as hemoglobin, insulin, ribonuclease and lysozyme, the primary structures of which are known, have not been as well studied in their other properties and have been rejected for the main study for various reasons. The classical protein foaming agents egg albumin and gelatin have been rejected because of uncertainty in composition and surface properties.

The difficulties in maintaining quiescent interfaces automatically makes difficult the study of any proteins which "coagulate" especially easily. Proteins in this class are egg albumin, hemoglobin, and to a lesser extent pepsin, and thus these were rejected for the main study.

Initial experiment with the apparatus showed that the solutions of protein which yield stable, flat, equilibrium lamellae gave foam films of large area when the solution was gently shaken, which is probably a manifestation of the surface elasticity necessary for stability. The proteins ribonuclease, pepsin, lysozyme and insulin foamed only over a limited pH range and were thus unsuitable for the main study.

Bovine serum albumin (BSA) and bovine- γ -globulin (BGG) were found to be the most suitable choices from foaming consideration; however, a literature search soon revealed the lack of knowledge of the necessary parameters required for analysis of the results for BGG. BSA has thus been mainly used for the present study.

RESULTS

The experimental method was checked with 0.05% solutions of a commercial sodium dodecyl sulphate. (It was not necessary, nor indeed feasible to use highly purified SDS, as this does not yield long lived black films). The thicknesses have been calculated

from the reflected intensities using the equivalent water thickness method of Mysels (83) with the correction of $13.5 \text{ \AA}$ for rigid films. The first black films of Perlin (84) were prepared with the addition of either KCl or LiCl. The latter only forms films of the first black state (85), whereas KCl can form both, depending on conditions. In this case, the second black film was formed from 0.1 N KCl solutions by removing the water normally used to saturate the top of the cell, thus allowing evaporation into the second black state (86). A comparison of the results obtained in this study with those of Mysels is shown in Table V.

TABLE V

Solutions (%)	Salt conc _n .	Saturation of air	Calculated thickness ($\text{\AA}$)	Mysels
0.05	0.1 N LiCl	Saturated	118	123
0.05	0.1 N NaCl	Saturated	112	117
0.05	0.1 N NaCl	Unsaturated	43	44

Preparation of solutions

0.1% solutions of BSA were found to produce the required stability in the apparatus, without excessive adsorption times being required. Observation of the films, however, showed that

there were small particles in the lamellae, causing them to be uneven in thickness. Observation of the solutions by means of a narrow beam of light showed the presence of some suspended matter (i.e. specks as well as diffuse Tyndall beam). Attempts were made to remove this by means of "Millipore" filters, but, unfortunately, the most inert filters (made of "nylon" or "teflon") are not available at pore sizes smaller than 1μ and therefore cellulose acetate filters had to be used. These were pre-treated by placing them in a Soxhlet extractor for one week and subjecting them to hot water. Surface active material was observed in the boiler water after a few hours and therefore the water was changed each day. After seven days the release of surface active material became very slow and as filtration was carried out in 5ml units taking only a few seconds contamination was minimal. Filtration was executed with a syringe attached to a filter holder by the "Luer-lock" device. The filter holder was of stainless steel and could be cleaned and steamed after use. A comparison of optical density of a stock solution of BSA at various dilutions with the same solutions filtered through 1000⁰A membrane filters is shown in Table VI.

TABLE VI

	<u>Optical density</u>		
Before filtration	0.490	0.580	0.754
After filtration	0.486	0.576	0.746

Attempts were made to determine the gain in weight of the membrane filters after 0.1% solutions had been filtered. A leached 1000⁰A filter was carefully dried to constant weight and a known volume of protein solution passed through it. Three volumes of water were passed through it to wash out any residual solute protein, and the filter was again dried to constant weight. Reproducibility was difficult to obtain between batches of BSA but typical values for the amount of insoluble protein were between 1.5 and 2.5% of the original weighed out sample. It would appear, therefore, that the insoluble matter consists of both proteinaceous and non-proteinaceous species. The results shown in Table VI at concentrations of 0.1% and above indicate that about $\frac{1}{2}$ to $\frac{1}{3}$ of the insoluble matter may be proteinaceous.

Even after filtration with the above-mentioned technique particulate matter was often observed at freshly formed interfaces. This was especially noticeable when films were formed in the holder after several previous films had broken. This observation suggested that the particles might have been formed as a result of compression at the interfaces and this was checked by observations on the light scattering properties of foamed solutions. A filtered BSA solution (0.1% or more) was placed in a tube in an "Eel" nephelometer and the sensitivity adjusted to a convenient value

on the galvanometer at as high a sensitivity as possible. The solution was then shaken in the nephelometer tube until a foam was formed. This was collapsed by evacuating the tube and releasing the vacuum suddenly. Several repetitions of this procedure completely removed the foam from the top of the tube and after one inversion the tube was replaced in the nephelometer and the reading on the galvanometer redetermined. Table VII shows successive readings after 3 foaming steps.

TABLE VII

Foaming steps	0	1	2	3
nephelometer reading	0	19	30	40

Agitation of surface layers of BSA thus produces solid matter which can break away and remain in suspension in aqueous solutions. This solid may be attributed to "surface coagulation" as defined for egg albumin. McRitchie and Alexander (87) observed a coagulum from spread monolayers of BSA on concentrated salt solutions at pressures around 20 dyne cm^{-1} , and it appears therefore that a high surface concentration of protein at relatively high surface pressures are the conditions for surface coagulation. For proteins adsorbing spontaneously from solutions, (e.g. egg albumin, pepsin etc.,) which coagulate readily, adsorption pressures must

be very close to a critical value for coagulation, and this may be exceeded locally on gentle agitation. For the other proteins, the critical pressure may be exceeded only by vigorous agitation. In the latter case, surface concentration begins to play a dominant part since any loss of segments of protein from the surface layer may be considered as a decrease in surface concentration. Proteins which are not usually considered as easily coagulable have lower surface areas at a given surface pressure. This may be interpreted in terms of collapse of segments of the protein molecule after initial surface denaturation. The best example of a non-coagulable protein is gelatin. This relatively hydrophilic protein collapses at the air-water interface (88) to give very low areas per molecule and it has not been observed to produce solid coagulum at this interface.

In order to remove the surface coagulum obtained during the formation of the lamellae surfaces, a very fine micro-pipette was drawn from glass tubing. This was attached to a vacuum line and used to clean the surfaces as one would the surface of a Langmuir trough. This operation was applied to both sides of the meniscus before the surfaces were brought together by suction. This procedure yielded films from 0.1% BSA solutions which appeared uniform to the eye when in the "black" state.

(i) Bovine serum albumin

a. Characterisation of the BSA.

The sedimentation characteristics of the protein in solution were determined in the Spinco^o model E ultracentrifuge at a speed of 59,780 rpm using schlieren optics. Photographs were taken at approximately 16 minute intervals after the front had moved away from the meniscus. These are shown in fig 12. The presence of a small amount of a faster sedimenting component can be observed and this was probably the BSA dimer (89). The actual amount was estimated by magnifying the photographic plates and calculating areas beneath the curve. All samples of BSA from Armour Ltd. showed between 2 and 5% of the faster sedimenting component. The sedimentation coefficient for the major peak was calculated from the relationship:

$$S = \frac{1}{\omega^2 x} \frac{dx}{dt} \quad \text{where } \omega = \text{angular velocity (radian sec}^{-1}\text{)}$$

$$\text{or } S = \frac{1}{\omega^2} \frac{d(\ln x)}{dt} \quad \text{where } x = \text{distance of solute boundary from centre of rotation.}$$

$$\quad \quad \quad x = 5.72 + r/M$$

$$\quad \quad \quad \text{where } r = \text{peak distance from reference point}$$

$$\quad \quad \quad M = \text{magnification factor for instrument}$$

Hence a plot of $d(\ln x)$ against t yields a straight line of slope.

$$\therefore S = \text{slope}/\omega^2$$

Table VIIa and graph **A** show the calculation and results for

Ultracentrifuge Study of BSA

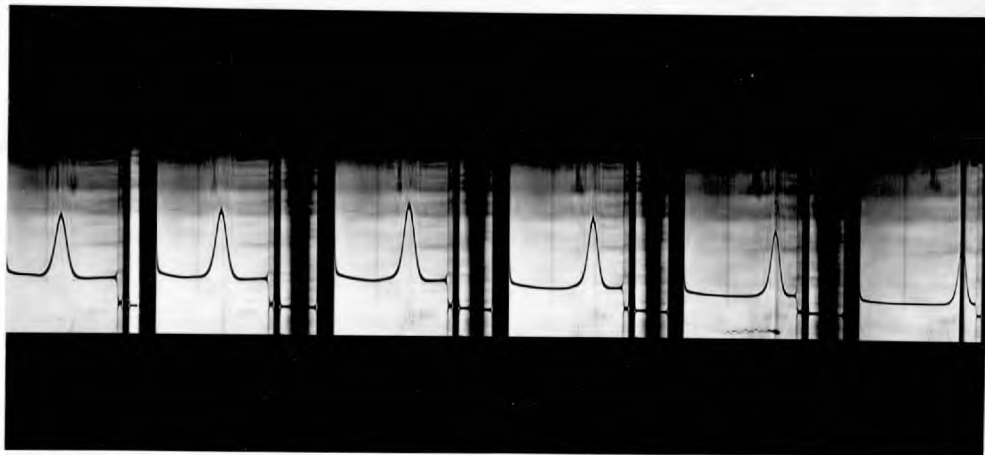


Figure 12

Reference (R)	Meniscus (M)	Peak (P)	M - R	P - R (x)	x/M(mm)	5.7 x x/M = X	$\log_{10} X$	t
27.715	30.507	35.798	2.792	8.083	0.3725	6.0925	0.78484	40
35.633	38.405	45.790	2.772	10.157	0.4681	6.1881	0.79156	56
25.109	27.875	37.321	2.766	12.212	0.5628	6.2828	0.79817	72
35.931	38.736	50.262	2.805	14.331	0.6604	6.3804	0.80482	88
5.719	8.507	24.459	2.788	18.740	0.8640	6.5840	0.81849	120
9.790	12.561	30.754	2.771	20.964	0.9661	6.6861	0.82518	136
18.026	20.820	41.316	2.792	23.290	1.0732	6.7932	0.83206	152
3.636	6.415	29.268	2.779	25.632	1.1812	6.9012	0.83891	168
9.261	12.040	37.314	2.779	28.053	1.2928	7.0128	0.84591	184

TABLE VII (a)
Calculation of S_{20} for BSA

Plot for calculation of sedimentation
coefficient of BSA

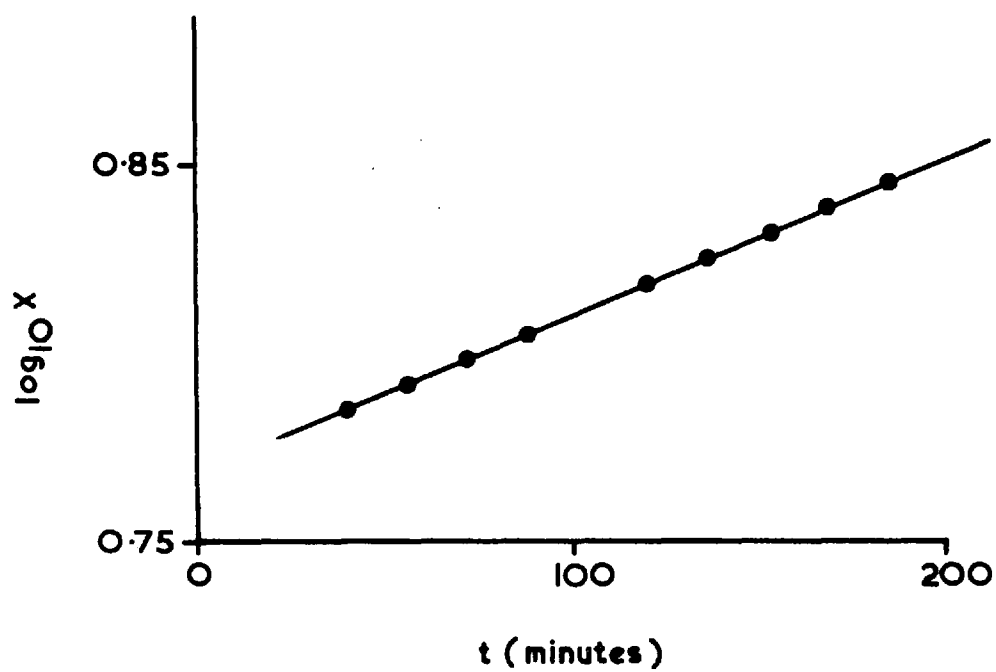


FIG.A

BSA yielding a value for S_{20} (the sedimentation coefficient for solution in water at 20°C) of 4.23×10^{-13} cgs units (or 4.23 Svedbergs) for a 0.8% solution. This value agrees well with the collected results reported by Johnson (90) which show an average value of 4.24 Svedberg over three independent investigations. Harrington et al (91) have also reported similar values. The BSA used can thus be compared to that of previous workers.

It has been reported that there is a fatty impurity in commercial BSA (92) which may be removed by allowing solutions at pH 4.0 to stand at 2°C , when the impurity may be centrifuged off. BSA defatted in this way yielded lamellae in the apparatus which differed in thickness from lamellae obtained from non-defatted solutions by only a few Angstroms, and results were always within the limits of uncertainty of the experiments on the non-defatted solutions. Studies were thus carried out on the non-defatted BSA.

The surface properties of the BSA were characterised by the surface pressure (π)—time (t) curves and also the π —area (A) curves for the protein concentrations and ionic strengths concerned in the study of free films. The results are shown in figs 10, 13. The π — t curves were determined by means of the hanging plate method and the π — A curves by the Langmuir-Adam floating barrier surface balance. Fig 13 also shows a

Π -A Curves for BSA

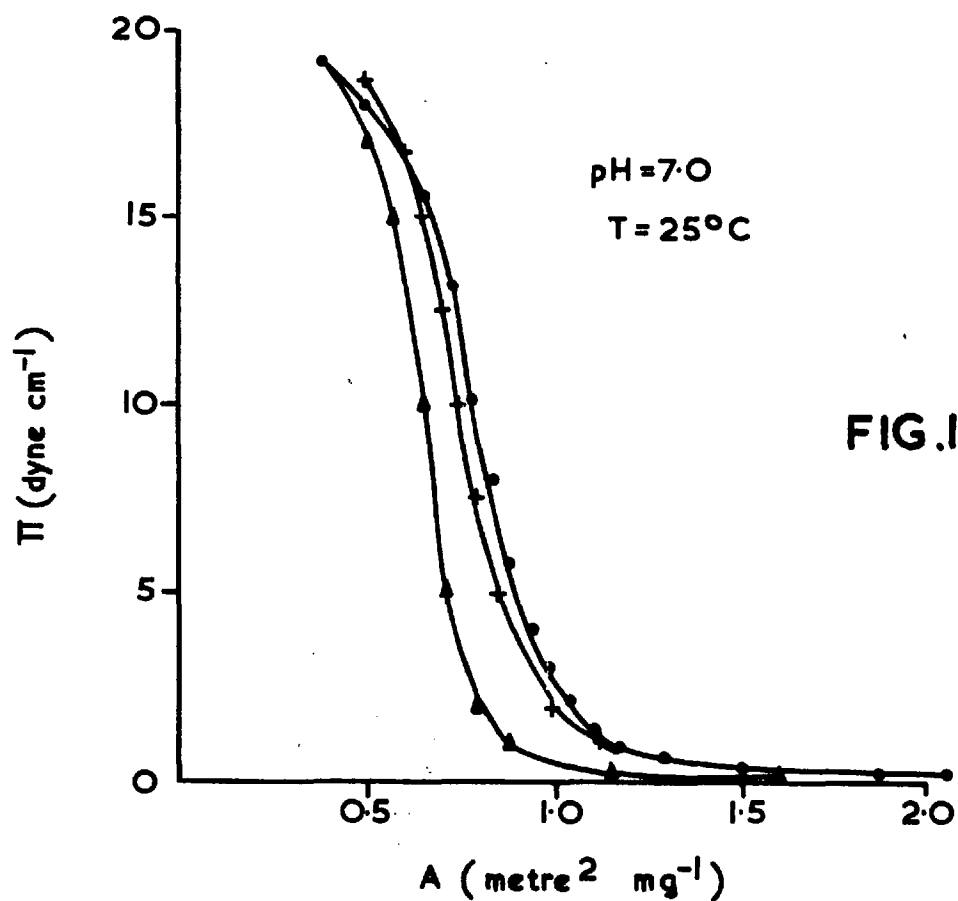


FIG.13

- B S A spread by Trurnit's method $I=0.1$
- ▲ B S A spread using Agla syringe only $I=0$
- + B S A spread using Agla syringe only $I=0.5$

comparison of the various spreading methods. The Trurnit method (93) was found to be the best for applying the protein into the interfacial region. The presence of 0.1 N KCl in the bulk solution had a small effect on both the π —t and π —A curves at the pH of the experiments. Thus analysis of results with and without KCl using the same parameters is not strictly accurate, but in the absence of accurate data on the various parameters required for the analysis, and since large variations are not to be expected in the region of 0.1 N $\frac{1}{2}$ KCl, comparison of the calculated thicknesses is probably valid.

The presence of a minor component in "Armour" BSA was also detected by means of electrophoresis on polyacrylamide gels using the discontinuous (disc) system described by Ornstein and Davis (94). Buffer solutions were prepared as described by Ornstein and Davis with the exception of solution (B) which consisted of:

1 M H_3PO_4	25.6 ml
2- Amino (hydroxymethyl) 1-3 propane diol (TRIS)	5.7 g
Water to	100 ml
pH = 6.85 - 6.95	

Electrophoresis was carried out overnight at room temperature and 15 mA. At the end of this time the gel cylinders were removed from their supports and stained with a 1% solution of

Discontinuous gel electrophoresis of BSA
(Applications of 50 μ g and 25 μ g)
Concⁿ of 1 mg/cc



Figure 14

Amido Black dye in acetic acid. The excess, unbound, dye was removed by rinsing in 7% acetic acid solution until the background gel was clear. A photograph of the results for BSA is shown in fig 14. The presence of a small amount of a minor component is clearly demonstrated. This is probably the dimer since gel electrophoresis separates both by size and charge. The relative concentrations could be estimated by density of staining, but since the method is not well established, this has not been tried.

(a) Major Study on BSA

The reported "limiting" values for the intensity of reflection are those at the point of bursting. A study was carried out to determine whether these values correspond to an "equilibrium thickness". At all pH's where sufficient stability allowed time for a scale expansion on the recorder to be executed (pH 5.7 to 8.5), the intensity of reflection slowly decreased with time. This was not observed with SDS and thus a true equilibrium thickness is not measured for protein solutions. Another observation substantiating this conclusion was that even in dilute solutions where stability is transient and the films were much more mobile in appearance, "black spots" were not evident. Thus protein films are fundamentally different to the solutions of SDS studied.

TABLE VIII

REFLECTIVITIES OF FILMS FORMED FROM
BOVINE SERUM ALBUMIN SOLUTIONS (0.1%)

$$(\lambda = 5470 \text{ \AA})$$

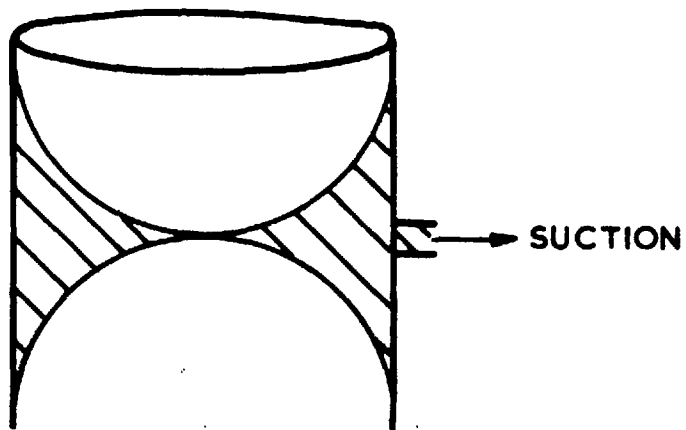
Surface layer thickness (D 1)			Homogeneous Approximations		18.0	12.5	15.3
Surface layer refractive index (FN 1)			1.333	1.50	1.44	1.44	1.47
pH	KCl conc ⁿ	I/I ₀ x 100	Thickness (Å)				
			1	2	3	4	5
2.0	0.00	Films unstable	-	-	-	-	-
2.0	0.10	Films unstable	-	-	-	-	-
3.0	0.00	More stability but not enough for study	-	-	-	-	-
3.0	0.10		-	-	-	-	-
4.0	0.00	0.18	98	62.5	-	85	80
4.0	0.10	Non-uniform films	-	-	-	-	-
5.3	0.00	0.17 ^a	95	61	79	82	78
5.3	0.10	0.05 ^a	52	33	37	39	36
5.7	0.00	0.28	122.5	78	106	109	107
5.7	0.001	0.25	116	74	99	103	99
6.0	0.07	0.058	55.5	35	40	43	39
6.0	0.10	0.05	52	33	37	39	36
6.5	0.00	0.178	97	64	81	84	80
6.5	0.005	0.124	81.5	54	65	69	65
7.0	0.00	0.212	106	67	90	93	89
7.0	0.05	0.068	60.0	38.5	45	48	44
7.0	0.10	0.050	52	33	37	39	36
7.0	0.30	0.77	-	~400	-	-	-
8.5	0.00	0.240	113.5	72	97	100	97
8.5	0.01	0.095	71	45	55	59	55
11.2	0.00	No limiting flat	-	-	-	-	-
11.2	0.10	(uneven)	Still thinning at point of bursting				

a - very fragile films

TABLE IX
EFFECT OF ADSORPTION TIME
0.1% BSA

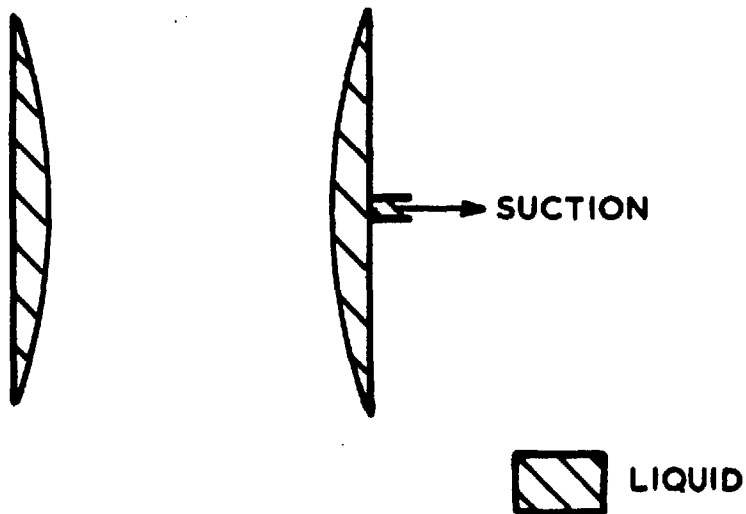
t(mins)	pH	KCl(N)	$I/I_0 \times 100$	$d \text{ (}\overset{\circ}{\text{A}}\text{)}$ FN = 1.50	Approximate Life-time (mins)
5	6.0	0.07	0.022	22	1
10	6.0	0.07	0.060	33	1
14	6.0	0.07	0.066	39	1
30	6.0	0.07	0.058	35	2
5	7.0	0.00	0.043	30	1
50	7.0	0.00	0.212	67	3 $\frac{1}{2}$

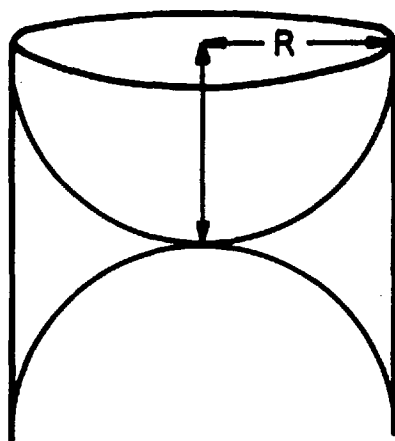
The ratios of reflected intensities to incident intensities for films formed from BSA solutions are shown in Table VIII. The ratios shown are averages obtained from at least three experiments on films with apparent plano-parallel regions extending to at least twice the diameter of the incident spot of light. Great difficulty was encountered in keeping the plano-parallel regions at constant radius, even with manipulation of the micrometer controlling the hydrostatic pressure. The radius of this region was always slowly changing and this suggests that the surface regions undergo change at constant hydrostatic pressure. The conclusion may be demonstrated by considering the surface area of (a) two hemispherical surfaces and (b) the same surfaces deformed into a cylindrical shape by complete flattening across the supporting orifice (fig 16). Since the flat region was often stable for up to



COMPARISON OF SURFACE AREAS BETWEEN HEMISPHERICAL
BUBBLE STATE AND BROKEN STATE

FIG.15

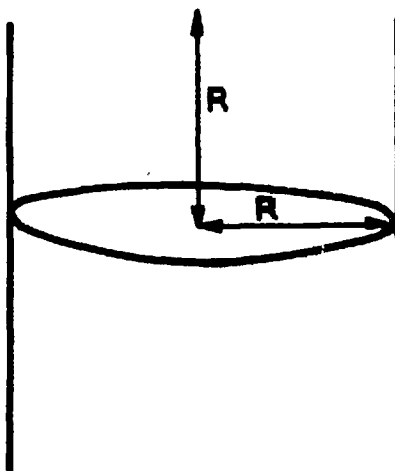




SURFACE AREA OF
HEMISPHERE = $2 \pi R^2$

COMPARISON OF AREAS BETWEEN HEMISPHERICAL
BUBBLE STATE AND TOTAL PLANO-PARALLEL STATE.

FIG.16



SURFACE AREA OF
CIRCULAR FLAT = πR^2

SURFACE AREA OF
CYLINDER WALLS = $2 \pi R^2$

TOTAL SURFACE AREA = $3 \pi R^2$

three minutes, the increase in surface area must be stabilised by stretching of the initial surface layers or by further adsorption as surface area becomes available, or more probably, by a combination of these two mechanisms. Because of the slow rate of adsorption of proteins, stretching will cause the overall surface thickness to be somewhat less than that at the adjoining hemispherical surfaces. This is further substantiated by observations on some films where the area around the flat region did not show a gradual increase in total thickness, but possessed areas of differing thickness. This behaviour was observed more often in films where adsorption times were long (i.e. ~ 45 minutes) from solution on 0.1 N KCl. The observation of uneven thickness in regions other than the flat strongly indicates stretching to an apparent even thickness. The stretching is probably compensated to some extent during the experiment by further adsorption and an absolute value for surface thickness cannot then be estimated. However, very thin lamellae must be stabilised by films with elastic properties; and since BSA monolayers show strong elasticity only at areas smaller than $0.8 \text{ m}^2 \text{ mg}^{-1}$, a minimum thickness can be estimated as about $12 \text{ \AA}$. The thicknesses of the various films have been computed for several values of surface layer thickness (d_1) and refractive index (n_1) as indicated in Table VIII. These

values have been chosen to represent reasonable values of d_1 and n_1 .

The smallest computed value for I/I_0 corresponds to a model of two surface layers touching each other. Of course, if this is greater than the smallest experimentally determined value, the model is invalid. Using this approach, surface thicknesses greater than $18 \text{ \AA}$, combined with refractive indices about 1.44, have been eliminated. Since 1.44 is considered to be a lower limit for the surface refractive index, the actual surface stabilising thickness (d_1) must be less than $18 \text{ \AA}$ for 0.1% solutions of BSA. The calculated total thicknesses have been rounded off to the nearest whole integer, except those where fortuitous choice of assumed n_1 , d_1 , and d_2 yielded exactly the same value for I/I_0 as that determined experimentally. Columns 1 and 5 may be compared by means of the correction suggested by Duyvis (95). Subtraction of the surface thickness used in calculating column 5 from column 1 yields the values shown in column 5 with an accuracy of $\pm 2 \text{ \AA}$ except at pH 4.0 and 5.3 where non-typical behaviour is encountered.

The observation that serum albumin thins to $100 \text{ \AA}$ or less in pure water indicates that the films are not stabilised by long-range double layer forces. The surfaces are therefore in

the primary minimum of the potential energy curve. Fig ~~1~~ 2 shows a graph of the theoretical attractive and repulsive forces for flat plates in the region of $100 \text{ \AA}$ separation for zeta potentials less than 25 mV ($e\psi_0/kT = 1$) at various values of the double layer thickness. There will be an energy barrier to the primary minimum when the attractive and repulsive curves cross each other and thus the surfaces either have a value of A greater than about 5×10^{-12} with a surface potential less than 15 mV ($e\psi_0/kT = 0.6$) or a lower value of A with a very low value of surface potential (i.e. 10 mV). The maximum values for observed ζ -potential on proteins, either adsorbed or in free solution, are about 30 mV at zero ionic strength (96). Values less than this are thus probably acceptable at the air-water interface since Abramson (96a) has reported that air bubbles in weak gelatin gels migrate with the same electrophoretic velocity as other particles in concentrated gelatin solutions. The difficulties involved in defining the slipping plane and position of charges for adsorbed proteins make the substitution of zeta potential for ψ_0 potentials dubious and values between 10 and 20 mV would appear plausible.

The value for A for water has been estimated at 6×10^{-13} erg (97) and determined at 7×10^{-13} erg for extremely dilute aqueous solutions of saponin, (which is a mixture of high molecular weight

glycosides containing hydrated sugar groups). It seems reasonable, therefore, to assume A to be of the order of 10^{-12} erg.

If the reasoning above is valid, then other stabilising forces must be operating, as discussed by Ottewill (98). There will be an osmotic pressure corresponding to a repulsion caused by the chemical potential change as the surface layers overlap, and also a resistance caused by any mechanical elastic properties of the layers. These extra repulsion forces will decrease the depth of the primary minimum and thus make the system more reversible. Reversibility of thickness was shown by the effect of reducing the hydrostatic pressure applied to films which caused a decrease in the area of flat film and an increase in thickness. It was not found possible to carry out the same experiment with the thinnest "black" films for by the time they had reached their "limiting thickness" they were very fragile and ruptured if an attempt was made to reduce the suction pressure. Theoretically the van der Waals pressure in an aqueous lamellae^o less than 100 Å thick is so much larger than the hydrostatic pressures applied externally that variations of suction would not be expected to affect the thickness of the black areas significantly. Thinning of protein films may also be caused by stretching of the surface layers. In the apparatus the formation of a

flat film causes an increase in surface area. The maximum increase is caused by complete flattening and is numerically equal to r^2 where r is the radius of the orifice (fig 16). For protein films, where repulsive forces do not appear able to keep the surfaces apart, the thickness of the stabilising protein surface layers contribute a large part of the total thickness and thus increase in surface area, followed by more efficient surface denaturation, would lead to a thinner film. Thinning caused by stretching is controlled by surface rheological properties of the surfaces. This may be demonstrated by the comparison of thinning curves obtained from solutions of BSA in water and 0.01 N KCl (Table X). The surface viscosities of BSA layers under these conditions have been taken from the collected results of Joly (99) and are compared with the thinning times for free films. In order to equate the surface areas it is necessary to increase the value of the surface viscosity on water and thus it is clearly demonstrated that high surface viscosities are paralleled by longer thinning times. Haydon (100) has shown that both surface viscosity and elasticity have a relationship to the stability of protein coated oil droplets and thus the observed relationship between viscosity and thinning time may only by a manifestation of an elasticity-thinning time interrelation.

TABLE X

	Thinning time (secs)	surface area $\times 10^{12}(\text{cm}^2)$	(Surface poises)
BSA in water	180	1.02	4
BSA in 0.01 N KCl	40	0.94	0.36

The approximate life-time of apparently flat films are shown in Table IX for several sets of conditions. Adsorption times greater than 30 minutes were necessary to produce the most stable films for which the spot of light could be moved around, and the focusing checked by maximisation of intensity on the recorder to yield results with a high confidence level.

An attempt was made to photograph a black film formed from BSA solution. This proved to be a very difficult task with the camera available because of difficulty in exact focusing and the extreme fragile nature of the film. (The action of moving the film to the next frame was often sufficient to cause bursting). Thus concentrations greater than 0.1% had to be used and thicker (silver), often irregular, films were observed. The results are shown in fig 17. In order to prevent change in film diameter during exposure, large films were formed which could not be evenly illuminated because of the natural stops in the microscope.

Free Films of BSA

(Showing (a) a dimple and (b) a central black area)

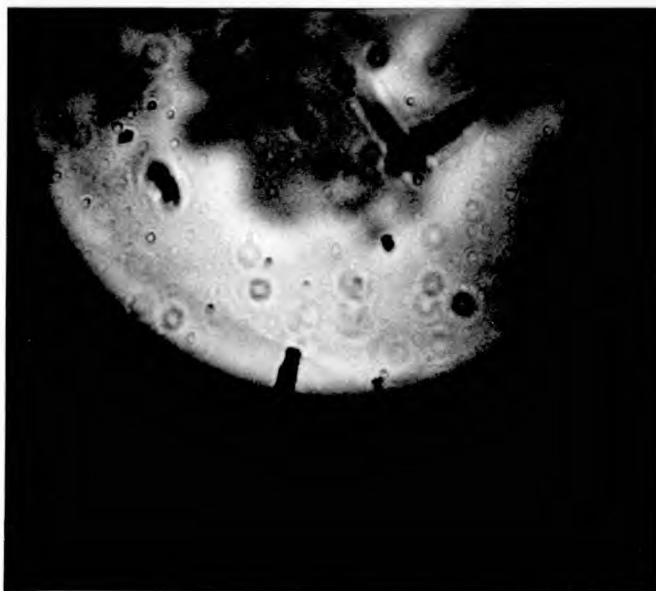
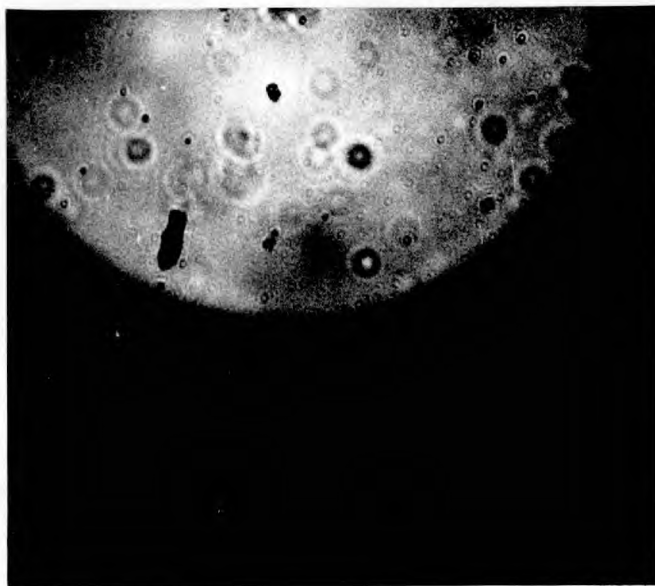
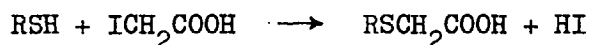
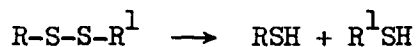


Figure 17

Therefore, one half of the film was illuminated, using a diffusing plate, and photographed; the presence of particulate matter in these films is clearly shown.

The unravelling of BSA at the air-water interface must be restricted by the 16 or 17 disulphide bonds (101) and the restriction has been studied by cleaving the S-S bonds by the method of Hunter & McDuffie (102). 8M urea was used instead of dodecyl sulphate and the sulphydryl groups were blocked with iodo-acetic acid to prevent re-association.



Urea was preferred to sodium dodecyl sulphate because of the very stable complexes formed by the latter with BSA. It is very difficult to remove the detergent from such complexes to yield the reduced BSA alone, whereas urea is simply dialysed out.

BSA was dissolved in 8M urea solution at pH 8.2 in the presence of sodium carbonate, and left for one hour. Sodium thioglycolate at pH 8.2 was added and the flask evacuated and filled with nitrogen. The flask was wrapped in aluminium foil and kept in a dark cupboard for 24 hours to prevent the formation of free I_2 . During this time the flask was evacuated and refilled with nitrogen twice. At the end of 20 hours a 1.25 fold excess

of 0.5M iodoacetamide was placed in the flask followed by evacuation and filling with nitrogen. 2N sodium hydroxide was used to bring the pH back to pH 8.2 while a stream of nitrogen played on the surface of the solution. The flask was left in the dark for 5 hours and then dialyzed 5 times against water at pH 9.0. A slight amount of precipitation was observed in the dialysis bag. The solution was then freeze-dried and stored in a refrigerator. Micro-Kjeldahl analysis showed the dried preparation to be 98% protein. Ultra-centrifugation at pH 10.0 showed a single peak which tended to diffuse with time (fig 18). It is probable that the preparation consisted of species in a small size range, or perhaps that the conformation was such that back diffusion took place for constant sized molecules; however, this is little understood. The pH was controlled at 10.0 to ensure complete solubility. The solubility characteristics showed hysteresis, a solution in alkali not precipitating until pH 5.2, whereas a solution titrated from acid solution was still cloudy at pH 7.5. For this reason films were studied only at pH's around 2.0 and 10.0. Long solution times (i.e. 12 hours) were required for pH's at say 3.5 and 8.5 and experiments were not carried out because 0.1% solutions were never obtained. Solutions foamed in the "insoluble" region,

Ultracentrifuge Study of S-S Reduced BSA

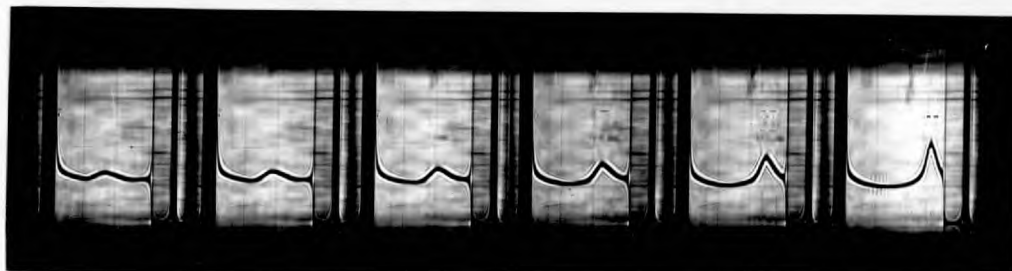


Figure 18

even after the solutions were filtered as for BSA. Filtered solutions showed no macroscopic insoluble matter when examined in a collimated light beam, but films tended to be of uneven thickness, even after surface cleaning by suction. Table XI shows the results for the approximately uniform thickness of films formed from 0.2% solutions of S—S reduced, carboxymethylated BSA. This concentration was necessary for sufficient stability for the study.

TABLE XI

pH	KCl Conc ⁿ (N)	$I/I_0 \times 100$	thickness (Å)	
			n = 1.5	n = 1.6
1.9	0	0.15	72	53
1.9	0.01	0.11	60	46
2.3	0	0.33	105	79
2.3	0.1	0.24	92	67
9.4	0	1.06	187	140
9.4	0.1	2.18 (?)	-	-
12.2	0	0.25	92	69
12.2	0.1	0.38	112	85

The thicknesses have been calculated assuming that the surfaces were in contact with a refractive index (n) of either 1.5 or 1.6. These will be minimum values unless the refractive

index is drastically altered by the S—S reduction and blocking. This is unlikely since refractive indices of proteins may be calculated from the molar refractivities of the amino acids and only a maximum of 36 out of some 630 residues are altered. Since the water binding properties of the reduced protein are unknown, the changes are probably not worth speculating about. In no case did the films reach a true equilibrium thickness, although the rate of thinning was slow by the time of rupture. If the hydrostatic pressure was suddenly increased during the slow thinning stage, the slope of the intensity-time curve changed abruptly and levelled off in a new slowly thinning state. The phenomenon is presumably^y due to the stretching of the surface layers as the flat area increased in size.

Non-ionic detergents with a zeta potential of about 10 mV (103) yield films of thickness close to twice the thickness of the hydrophobic part of the stabiliser molecules and analysis of protein films in terms of thick surface layers would appear justified.

Experiments have also been carried out on solutions of BSA reduced by sulphite in 8 M urea actually using the urea solution in the cell. The aqueous urea solutions were saturated with nitrogen before reaction in order to remove any possibility of re-oxidation. Uneven films were obtained over the pH range 2 to 9

with films often stable for up to 2 hours, providing about 45 minutes adsorption time was allowed. Hazy solutions were obtained between pH's 4 and 5, and, therefore, this range was avoided. At pH 6.2 the films thinned slowly until a black spot appeared at the centre of the flat. This appeared homogeneous to the eye and had 0.11% reflection, corresponding to a thickness of at least $45 \text{ \AA}$, as calculated for a refractive index of 1.6. Around the edge of the black region were areas of irregular thickness and the apparent flat area appeared to have been stretched out of these. Many films were obviously inhomogeneous even after filtration of the solution and surface cleaning by suction, indicating ease of surface coagulation.

Discussion of Results for BSA

The native molecule of BSA changes its dimensions with pH (104), but over the range in which stable films were obtained the minimum major axis length is about $30 \text{ \AA}$ to $40 \text{ \AA}$ for the model of an ellipsoid of revolution. The minimum thickness of two layers of native molecules would thus be $60 \text{ \AA}$. The most reasonable estimated thicknesses from the intensity of reflection in 0.1 N KCl are $35 \text{ \AA}$, with an estimated confidence limit of $\pm 10 \text{ \AA}$. The unravelling of the molecule is thus amply demonstrated. Consideration of the thickness measurements leads to the conclusion

that the charge on the protein surfaces is insufficient to keep the surfaces apart, and therefore an explanation of the variation of thickness with ionic strength is required. As mentioned throughout the results section, black films were observed momentarily for solutions up to 0.1% if short times were allowed for adsorption to the surface. These were, however, too unstable for accurate study. Longer adsorption times yielded more stable films but of irregular thickness around the periphery. It was as if the thin central region "squeezed" out the thicker layers as it was formed. This indicated either that the surface layers consist of components of different thicknesses with one or more of the species capable of changing shape as thinning proceeds, or that it contains uniform species capable of being stretched. Consideration of the adsorption process appears to support the former since the first molecules to arrive at the interface have a large area in which to unravel, whereas those arriving later have to force their way into the surface region against the surface pressure of the first molecules. According to McRitchie (105) adsorption proceeds as long as a critical area of surface is available for, or can be cleared by, the impact of a molecule approaching the surface. It seems reasonable to assume, therefore, that those arriving last will only just be

able to clear this critical area, the rest of the molecule remaining in a more native form. Thus the surface layers appear to be of uneven thickness. If, as appears the case, the surfaces are "touching", extra applied thinning pressure causes an increase in surface area, which may be covered by both unravelling of the more native molecules at the surface and stretching of the unravelled molecules under the thinning forces. Rupture then occurs when a large area of the thinner molecules is produced and the ripples proposed by Vrij (106) are capable of tearing the film locally. This would explain the dependence of stability of protein stabilised films on the various surface rheological parameters (107).

Molecular rearrangement on breaking the disulphide bonds is evident from the insolubility range conferred upon the protein. It is reasonable to assume that the molecules of modified protein in bulk solution at pH 2 and 12 are also in a form different from the normal native molecule. The thick layers that are formed might arise in one of two ways:

- (a) the molecules are large and do not unravel to any great extent
- or (b) the initial molecules unravel to a greater extent than the native molecules and are

then collapsed by the pressure built up from molecules arriving later at the interface, giving an overall thick film.

At pH 12, conditions will be such that unravelling of the molecule is favoured since the molecular restraints are removed and the molecule is charged. The extra charged groups at alkaline pH will make unravelling more likely than at acid pH, but in both cases the hydrophobic side chains of the protein molecules may be removed from the aqueous phase by adsorption. It thus seems possible that the thickness is caused by surface collapsed material together with molecules in a relatively native state from later adsorption. The life-time for films of the S—S reduced BSA was often over 1 hour for films aged as little as 15 minutes. The film was always slowly thinning and this indicates a greater capacity to assimilate the stretching forces applied to the surfaces. This is most easily envisaged if collapse is considered, since a film consisting of molecules which do not unravel at the interface will not contain molecular tangling with which to resist the stretching forces.

Films from both BSA and S—S reduced BSA may thus be analysed in terms of mixed films of partially unravelled molecules and nearly native molecules. For native BSA the disulphide bonds

restrict the unravelling and thus the resilience to shock.

In the reduced form the extra freedom conferred on the molecule allows thicker films to be formed by collapse. As the surfaces are subjected to local or sustained disturbances, the loops of collapsed out material and the relatively undenatured molecules rearrange to take up the exposed surface areas and thus confer long stability times in the thin films.

Multilayers do not appear to be present for solutions of up to 0.1%. This is not surprising since only a small amount of dimer appears in fresh solutions of BSA, indicating weak forces of interaction. Multilayers may, however, be the cause of the thicker, more uneven, films obtained from solutions of concentrations greater than 0.3%. This sort of behaviour has been described by Bull for egg albumin (108).

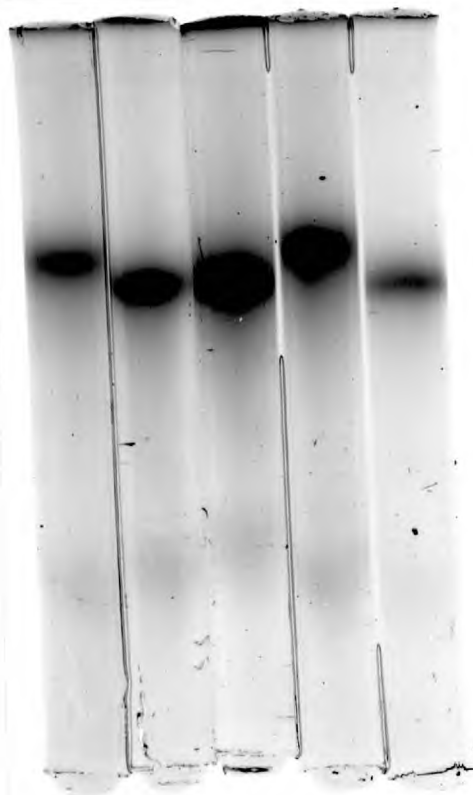
(b) Bovine γ -globulin

Bovine γ -globulin (BGG) was characterised by its sedimentation and electrophoresis characteristics. The ultracentrifuge pattern showed two components, one at 7S and the other 9S, the latter being 8% of the total content as judged by square counting. The "disc" electrophoresis on polyacrylamide gels also showed two components. Comparison of fig 14 with the results for BSA (fig 19) shows the minor component to be of a different nature

Discontinuous Gel Electrophoresis of γ -Globulin
(1 mg/cc applications of 25 μ l and 50 μ l)



After 90 Minutes



After 4 Hours

Figure 19

to the BSA dimer, even though dimers of γ -globulin are reported in the literature (109).

0.1% solutions of bovine γ -globulin were found to give sufficient stability for study. Solutions were filtered through 1000 Å "Millipore" filters and the surfaces of the films cleaned by suction, as for BSA. Visual observation of the films made it immediately obvious that BGG films were thicker than those from BSA. No films ever thinned to less than the grey state unless the surfaces were brought together by extreme hydrostatic pressure (e.g. 3 cm H₂O) which conferred adsorption times of less than 2 minutes. These films were, however, not stable for long enough to obtain reliable results for thickness. The results obtained are shown in Table XII, and Table XIII.

TABLE XII

pH	KCl conc ⁿ (M)	I/I ₀ x 100	Thickness (Å)	
			n = 1.6	n = 1.5
2.0	0	Insufficient	-	-
2.0	0.1	stability	-	-
3.2	0	Insufficient	-	-
3.2	0.1	stability	-	-
4.4	0.1	0.5	85	105
5.7	0	Cloudy solution		
6.0	0	1.13	127	157
6.0	0.1	1.18	129	161
7.0	0	0.89	113	139
7.0	0.1	1.10	125	155
11.0	0	Insufficient		
		stability	-	-
11.0	0.01	Insufficient		
		stability	-	-

TABLE XIII

t (mins)	pH	KCl	$I/I_0 \times 100$	n = 1.50	Approx. life-time (mins)
2	6.0	0	0.60	115	1
5	6.0	0	0.78	131	18
20	6.0	0	1.13	157	60
5	6.0	0.1N	1.18	155	10

The films invariably contained areas of greater thickness than the average which were slowly pushed to the periphery as thinning continued. All reported thicknesses are for the central area when devoid of visual inhomogeneity. None of the films ever reached a constant thickness, an observation, which, combined with the squeezing out of the thicker areas, again strongly suggests surface stretching. The results have a variance of $15 \text{ \AA}$ and it appears highly probable that the spot of light yielded average thicknesses for the area of incidence. This is further indicated by measurements of thickness at different points of the film. Variations of $5 \text{ \AA}$ thickness were apparent for relatively small movements of the light spot, even though visually the areas looked identical in intensity.

Discussion

The overall size of the "globulin" class of proteins is greater than that of the albumin. γ -globulin has a molecular weight of about 160,000 and consists of four separate polypeptide chains joined by five S—S bonds. Its isoelectric point is 5.7 for

bovine varieties, but differs if obtained from other animal sources. γ -globulin (source unspecified) has been reported (110) to consist of a dense core of dimensions $240 \overset{\circ}{\text{\AA}} \times 24 \overset{\circ}{\text{\AA}} \times 19 \overset{\circ}{\text{\AA}}$ surrounded by a loose shell, the dimensions of which are difficult to determine by low angle X-ray scattering on solutions. The core contains about 75% of the mass and a crude estimate of the smallest overall dimension is about $40 \overset{\circ}{\text{\AA}}$. Therefore, two layers of native molecules do not account for the observed thickness unless the molecules are steeply oriented to the plane of the surface. However, simple adsorption of a protein in its native configuration seems very unlikely as in solution it will present mainly hydrophilic groups to the environment and this configuration gives no reason for adsorption. The surface pressure-area curves for γ -globulin have been determined by Demeny et al (111) and show a very low limiting area at pH 6.0. This seems to indicate a form of collapse, especially as change of the pH of the subphase causes more complete spreading. The thick layers may be caused by a general unravelling followed by a collapse of the loose outer shell to the extent of some $30 \overset{\circ}{\text{\AA}}$, and perhaps by some of the core as well, but only to the extent allowed by the S—S links between the four chains (112). A definite interpretation must, however, depend on a full study of the surface properties of this protein which has not yet been carried out.

PEPSIN

Pepsin was characterised on the ultracentrifuge and found to have only one component with an S_{20} of 1.98S. This is in good agreement with the results of Edeloeh (113). At pH 5.6 the molecule may be described by an ellipsoid of revolution of axes $74 \text{ \AA}$ and $37 \text{ \AA}$ and it is to be expected that at pH's close to the isoelectric point (about pH 2.0) the diminished charge would cause a contraction in molecular size.

Results

0.1% solution of pepsin yielded uneven films which contained coagulated areas so thick that often the area surrounding them showed interference fringes. Therefore 0.05% solutions have been used in this study. The results have been analysed in terms of a homogeneous model of refractive index 1.5 (114).

TABLE XIV
0.05% Pepsin

$\lambda = 5470 \text{ \AA}$

pH	KCl (M)	$I/I_0 \times 100$	Thickness ($\text{\AA}$) $n = 1.5$	Life-time
2.0	0	-	Little stability	
2.0	0.1	-	Little stability	
2.2	0	0.10	46	1 min
2.2	0.1	0.18	62	
2.3	0	0.12	50	
2.5	0	0.38	90	15 mins
2.5	0.01	0.23	70	
2.6	0	0.28	77	
2.9	0	0.54	109	10 mins
2.9	0.01	0.32	82	
3.1	0	0.15	57	1 min
3.4	0	-	Little stability	
3.4	0.01	-	Little stability	

The films were very resilient to changes in hydrostatic pressure i.e. changes in the diameter of the thin film. However, excessive mechanical disturbance of the surfaces caused the appearance of semi-solid matter in the films similar to that found in films from 0.1% solutions.

Films over the whole stability range were plagued with the presence of this solid and much more attention had to be applied to cleaning by suction to obtain films homogeneous to the eye. Kronman (115) has observed that solutions of pepsin have anomalous light scattering properties if shaken. This was checked, as for BSA, using the nephelometer. Typical results are shown in Table XV (the light scattering being expressed in arbitrary units). These strongly support the idea that pepsin coagulates readily at the air-water interface in the pH range studied here.

TABLE XV

pH 2.5 0.1% Pepsin

Number of consecutive Foamings	0	1	2	3	Solution refiltered
Nephelometer reading	27	45	65	70	25

Visual observation of the solutions in the light beam showed white strands of material present. The filtration of the final solution removed these and a light scattering value corresponding to a lower protein concentration was obtained.

None of the films were allowed adsorption times longer than 10 minutes for film thickness measurements because of the occurrence of irregularities, and thus the results expressed as thicknesses have little significance. However, at comparable adsorption times the results in the high stability region are in accordance with observations of Cumper and Alexander (116) who deduced that the thickness of the surface films decreases with ionic strength. Three experiments were carried out in the region of relative instability in an attempt to follow the rate of increase of thickness with time. The results are shown in Table XVI for solutions at pH 2.3.

TABLE XVI

0.05% pepsin pH 2.3

<u>Adsorption time</u>	<u>$I/I_0 \times 100$</u>
15	0.05
30	0.06
40	0.11

At this pH the films were apparently more nearly homogeneous, but not as stable as those at pH's in the range 2.5 to 2.9. The results clearly show the build up of thickness with adsorption time. Each of the films had reached a slowly thinning state stable for about 1 minute, and in the absence of reliable surface rheology data little analysis of these results can be carried out.

Discussion

Pepsin is a proteolytic enzyme consisting of amino acids in a single polypeptide chain (117). It has a molecular weight of 34,500 (118) both in bulk and at the air water interface (119). It consists of 341 amino acid residues (120) of which 60% are non-polar, 71 are acidic in nature, 36 free hydroxyls and only 4 are basic (one lysine, one histidine and two arginines). The molecule contains 3 S—S bonds and its isoelectric point (if it really has one, which has been queried) is around pH 2.0 (121). Optical rotatory dispersion shows the α -helix is not a characteristic of pepsin (122). It has been suggested that the molecule is packed into a compact globule by the hydrophobic interaction of the non-polar amino acid residues (123).

Pepsin has been demonstrated to spread on water under favourable conditions at pH 6.0 (124) and 2.0 (125) as judged by the large areas obtained in π —A curves. However, the measurement of π —C curves (C = surface concentration) by applying solution to an air-water interface at constant area showed completely different behaviour (126). The surface pressure rose only to the order of 5 dyne cm⁻¹ at very high surface concentrations, whereas the π —A curves showed pressures of over 20 dyne cm⁻¹. This behaviour has been interpreted (127) in terms of surface layers of species

in various states of unravelling (128). The extreme variation of apparent thickness with time of adsorption, and the ^{ease} ~~ease~~ of formation of surface coagulum indicates a flexible model where denatured molecules can rearrange and the more native molecules unravel further.

LYSOZYME

Lysozyme was checked for homogeneity in the ultracentrifuge and because of its limited range of action not further characterised. Its S_{20} , was 1.99 at pH 11.9 in phosphate buffer.

It has been amply demonstrated by Hamaguchi (129) that lysozyme does not unravel to any great degree at interfaces where the pH of the aqueous phase is below 10.5. This is paralleled in the present study by finding that the films were unstable up to pH 11.0 for 0.1% solution. Films at pH's above 11.0 could be thinned to semi-stable homogeneous flat areas only if adsorption times less than 5 minutes were used. Higher adsorption times led to early breakage of uneven films. The films were not resistant to sudden changes in surface area.

Old solutions (i.e. $\approx$ 12 hours) foamed when shaken, giving stable lamellae of large area, which visibly thinned to a dark grey colour over quite large areas. Films from old solutions thinned to 80 ⁰ Å or so, as analysed on a homogeneous model, there

being little variation with ionic strength. Variation of molecular state was checked on the ultracentrifuge but no difference was observed. This does not, however, rule out the breaking off of a small amount of a small molecular weight component (perhaps by bacterial action) which aids in stabilisation.

Films from solutions below pH 10.5 in 8M urea were not stable despite the fact that urea aids spreading. The addition of 0.03 M sodium sulphite to the urea solution conferred stability on the films but produced visible irregularities. Shaken solutions produced solid matter visible to the eye, and the foams produced from the urea/sulphite solutions were often stable for 36 hours.

Discussion

This enzyme has its iso-electric at 10.5 and thus is positively charged at all pH's lower than this. The complete primary and secondary structure is known and has been reviewed by Phillips (130). The molecule possesses α -helical regions, random coil regions and regions stabilised by strong hydrophobic interactions.

The results indicate that unravelling of the molecule is necessary for stability and, furthermore, the greater the capacity for unravelling the more stable are the films (and foams). Lysozyme monolayers are in their most expanded state when the five disulphide bonds are broken (131) but in films formed from sulphite and

urea solutions thick films were observed and thus unravelling over the whole stabilising film is ruled out. Ultracentrifuge measurement on urea solutions is complicated by non-constant base lines, and thus the molecular state could not be checked. No attempt was made to isolate the carboxymethyl derivation of S—S reduced lysozyme because the film irregularities (as shown for S—S reduced BSA) complicate accurate analysis. The extra spreading indicated by monolayer work probably indicates a greater degree of flexibility in the molecule and this allows collapse of spread parts of the surface layer to produce an overall thick proteinaceous surface.

The observed instability of solutions of lysozyme in water at high adsorption times is difficult to understand unless the surface rheological properties take some time to build up the final state. If these possess rigid character then the behaviour may be explained.

The stability at low times might then be due to the Gibbs-Marangoni effect, but all stabilities above pH 10 are liable to analytical complications because of the lability of S—S bonds and the resulting possibility of polymerisation, and thus speculation is unwarranted.

INSULIN

0.1% solutions of insulin were found to foam in the pH range

2.0 - 10.5. However, solutions were hazy between pH 3.8 and 7.0. In very acid conditions clear solutions were obtained but in more alkaline conditions haziness was still evident. Experiments were carried out successfully in water at pH's 2.3 and 10.3 if adsorption times of 40 minutes or greater were allowed. Such films thinned very slowly and burst at reflectivities around 0.4%, indicating thick surface layers. The films thinned continuously and did not yield equilibrium flats even though the rate of thinning was very slow. Films formed from surfaces which had been previously burst (without extra cleaning) yielded stability after only 10 minutes ageing time. These burst at thicknesses around 1% reflectivity, suggesting that thick layers caused by mechanical disturbance can be advantageous to the stability of insulin films. Well cleaned films were, however stable only for minutes.

Addition of KCl to insulin solutions at pH 2.3 and 10.3 increased the stability of the films; flat areas were often stable for more than 1 hour. Such films, however, were still thinning and any sudden increases in hydrostatic suction caused a sharp increase in the rate of thinning. The thickness settled very quickly to a lower value which again gently thinned. For example, an increase of hydrostatic pressure from $1\frac{1}{2}$ cm of water to 5 cm water decreased

the reflectivity from 0.65% to 0.3%, the area of the flat region meanwhile increased sharply.

TABLE XVII

0.1% Insulin solutions

$$\lambda = 6328 \text{ \AA}$$

pH	KCl	$I/I_0 \times 100$
1.2	0.1	0.09 transient stability
2.3	0	Poor average because of low stability
2.3	0.01	0.65
3.8-7.0	0	Not enough protein in solution after filtration to confer stability
8.0	0	Uneven films with grey patches
9.0	0	
10.3	0	0.39
10.3	0.1	0.34

Discussion

Even though insulin was the first protein to have its primary structure completely characterised, its surface properties are not well known. This is probably because even the insulin monomer consists of two polypeptide chains (joined by two S—S bonds) which makes analysis difficult. The observed insolubility of the insulin agrees with the results of Fredericq and Neurath (132), being caused by the various polymeric forms which insulin takes up as the pH is made more alkaline (133). At pH 1.2 and 10.3 insulin is in the monomer form with a molecular weight of 6000 (134), whereas at pH 2.3 it is in the dimeric form (135). This is reflected

in the limiting thickness at pH 2.3 being greater than for other pH's. It is difficult to assess the degree of unravelling, if any, that occurs on adsorption, since the size of insulin molecules is unknown. Even the X-ray structure of the insulin crystals is the subject of much debate (136). However, Fredericq has demonstrated that the monomer has an axial ratio close to unity and is thus almost spherical.

Insulin has been reported to obey the Gibbs adsorption law (137) at pH 2.0 for concentrations between $5 \times 10^{-4} \text{ g l}^{-1}$ and $3 \times 10^{-2} \text{ g l}^{-1}$, but since the concentration necessary for convenient study was above this range, the possibility of reversible denaturation or adsorption in the truly native state is unlikely. The observation of sudden thinning on increase of the hydrostatic pressure strongly suggests a thinning against strong surface rheological forces and must infer surface stretching. The greater stability of the film in 0.1N KCl indicates a change in the rheological properties from those formed from HCl solutions alone. This is supported by the observations of Harrap (138) who showed marked changes in surface potential and pressure with increase of ionic strength. If this is caused by a greater unravelling of the molecule then the Gibb's Law is less likely to apply.

Synthetic Polypeptides

(a) Poly-glutamic acid

Poly-L-glutamic acid was not soluble below pH 4.5 (139). Optically clear solutions above this pH yielded very unstable films and only transient foams up to concentrations of 0.2%. Solutions made at neutral pH and then adjusted to below pH 4.6 became cloudy and yielded more stable lamellae. These were uneven in thickness and suggest a sort of solid stabilisation mechanism. The addition of KCl at pH's around 4.6 increased the overall stability of lamellae but at the same time caused more inhomogeneity in them.

(b) Poly-L-Lysine

Poly-L-Lysine was soluble over the pH range 2 to 12.5. No stability was conferred on lamellae for pH's up to 10.0 and concentrations up to 0.2%. Transient films were formed at pH 11.5 with a stability of a few seconds only and a bursting thickness of the order of $100 \text{ \AA}$.

Copolymer L-phenyl alanyl-L-Lysine HBr (1 : 1)

This copolypeptide was soluble only in water below pH 7.8, though difficulty was encountered in obtaining 0.1% solutions. 0.05% solutions were used, forming films which burst below pH 7.8 at reflectances in the region of 2%. Solutions in the "insolubility"

range yielded more stable foams and films even after filtration by a 1000 Å^o filter. After such filtration black films were obtained, but not of sufficient stability for study. At pH 8.0 filtered solutions produced mobile films, whereas unfiltered solutions yielded more rigid and uneven films.

Copolymer-L-phenyl alanyl-L-Lysine HBr (2 : 1)

Unfortunately this copolypeptide was soluble only to about 0.04% at neutral pH and thus little film stability was obtained. However 0.01 g in 10 cc were left in a refrigerator for 48 hours at 5°C in water adjusted to pH 2.3 and a solution at 0.06% obtained, which yielded films with more stability than for the 1 : 1 copolymer as shown in Table

TABLE XVIII

	<u>Stability time (secs)</u>
Copolymer 1 : 1	3
Copolymer 2 : 1	10

A dispersion of the 2 : 1 copolymer made by placing insoluble particles at pH 6.0 in an ultrasonic bath yielded more stable films and foams than did the true solutions, though life-times were still only of the order of 20 seconds.

Discussion

The foaming results obtained from the synthetic polypeptides in their insolubility ranges suggest that stability is often enhanced

if the surfaces of films are covered with solid or semi-solid matter in a manner similar to that which causes stabilisation of emulsions (140). For foams the forces acting on the stabilising layers must be great enough to disrupt the stabilising layers and cause the very low stability times.

In no case was the balance between bulk solubility and surface stability obtained, and it appears that a much more complex polypeptide is required for a good protein analogue. The only previous studies at the air-water interface on any of these polymers are those of Isemura and Hamaguchi (141) on spread monolayers of poly-L-glutamic acid. These authors clearly showed that poly-glutamic acid collapses very readily above pH 4.6, the surface viscosity only rising to very small values. Coherent adsorbed films would thus appear improbable.

The Caseins

Casein is the major component of milk protein, constituting about 80% of the total protein content (142). In its natural state this protein exists as micelles of average diameter of 80 to 100 m μ . Three major electrophoretic components may be separated by chemical means and are designated α, β, κ . The relative amounts of these components are estimated at 55% : 35% : 15% (143). There

is much confusion of the question of homogeneity of the various fractions and, at present, the fractions are best defined by the method of preparation.

α -casein

Milk from Ayrshire cows contains specific genetic variants of the various components. It contains only the α_s -B variant of α -casein, where α_s -casein is defined as the component of α -casein insoluble in 0.4 M calcium chloride at pH 7.0 and 4°C; it is stabilised by the κ fraction against calcium precipitation. This fraction has the fastest mobility of the three components considered here, and the α_s -B component has been prepared by the method of Thompson and Kiddy (144).

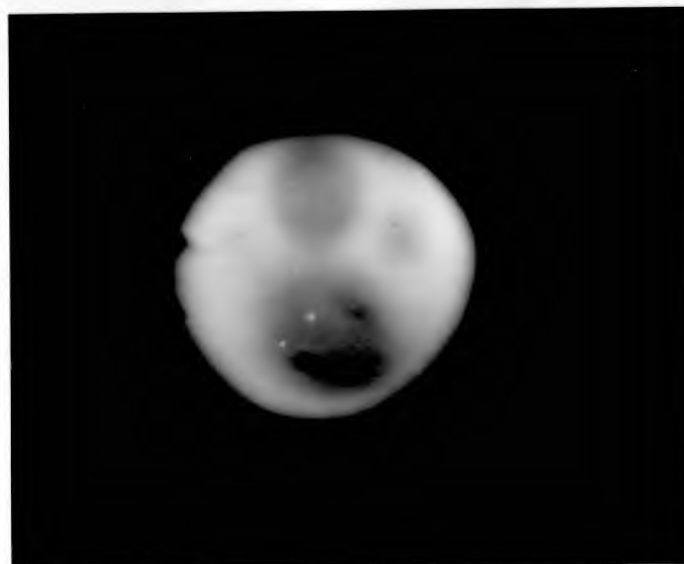
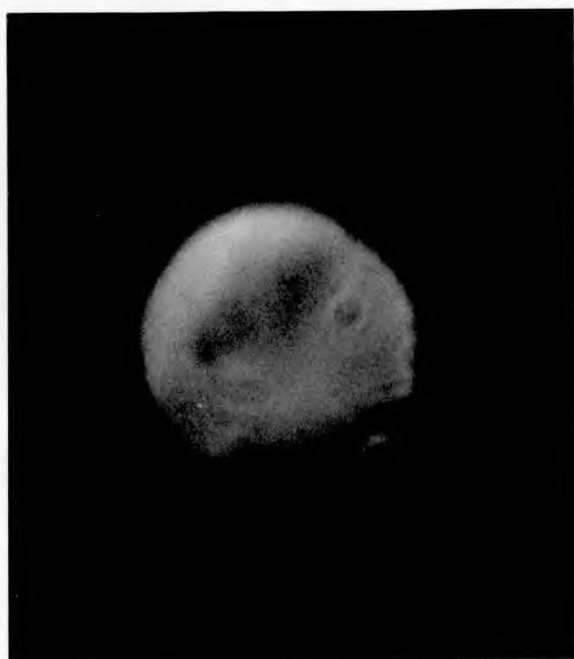
Results

Little stability was found for apparently even films formed from solutions with concentrations up to 0.1% over the pH range 2.0 to 10.5. Addition of 0.1 N KCl had little effect except for long ageing times at pH's near neutrality. Usual behaviour was for bursting to take place while the film was still thinning quite quickly.

β -casein

This fraction is defined as the component of whole soluble casein, soluble in 4.6 M urea, but insoluble in 3.3 M urea at

β -Casein Films



Figures 20 and 21

pH 4.6. The β -A variant has been separated from Ayrshire milk using the DEAE cellulose chromatographic method of Thompson and Pepper (145).

Results

Solution of this component was very difficult below the iso-electric point, and filtered solutions did not contain enough protein to yield stable flat lamellae. At pH 5.5 and above, a transient stability was obtained which was immediately increased by the addition of KCl. In 0.1 N KCl stable films were obtained which were somewhat uneven in thickness, an average value of $90 \text{ \AA}$ being measured as a minimum possible thickness for homogeneous films of refractive index 1.6. The flat areas of β -casein films were very resistant to change in surface area, thus allowing small lamellae to be photographed. Photographs of β -casein films formed from an 0.1% solution in 0.1 N KCl at pH 5.7 are shown in fig 20. The very slow appearance of the black area is shown in fig 21.

κ -casein

The κ -casein fraction may be defined as the component of a complex with α -casein which is soluble in 0.4 M CaCl_2 at pH 7.0 and 4°C . It is the component which is attacked during the action of rennin on whole casein (146) and is a glyco-peptide in nature. The κ -casein used in this study was prepared by the method of Hill (147) with purification on DEAE cellulose.

0.1% solutions were obtainable at pH 2.0 and above pH 5.0. The films obtained were not homogeneous in thickness, even in the presence of KCl, which was necessary for stability at pH's greater than 5.0.

Results

TABLE XIX
0.1% κ -casein films
($\lambda = 6328 \text{ \AA}$)

pH	KCl (N)	$I/I_0 \times 100$	Comments
2.0	0	1%	very uneven
2.0	0.1	0.8%	very uneven
6.0	0.1	0.84%	uneven
8.0	0.1	0.75%	uneven
10.0	0.1	0.61%	uneven

Above pH 5.0 the results indicated a minimum possible average thickness of around $100 \text{ \AA}$ and observation of the films under white light showed the colour to be grey.

An increase in the light scattering properties of films at pH 2.0 in 0.1 N KCl after foaming was demonstrated on the nephelometer as for previous proteins, and this provides an explanation of the very uneven thickness.

Discussion on the caseins

The results show that stable thin films of the casein fractions are dependent on the presence of quite large amounts of KCl, except perhaps for α -casein, where stability is always low. This immediately suggests that molecular association of the protein molecules is connected with the stability since the various fractions are known to polymerise in salt solutions (148). Of the three fractions, α -casein is the least prone to association especially at pH's above 7.0 (148). However, since the literature observations were always carried out in buffer solutions, a comparison might be invalid, especially as the type of buffer has an effect on the degree of association. At 25°C α -casein is associated only to a small degree, the sedimentation coefficient rising from 1.55 to 4.24, and there is little likelihood of either β - or κ -caseins being monomeric (148). The results might thus be explained in terms of molecular size i.e. surface thickness. This is not unreasonable since the electrophoretic mobilities of the casein fractions are low (149), and yield calculated zeta potentials of less than 12 mV. The caseins would be expected to yield thick layers since a frictional coefficient ratio has been determined at about 1.9 for each of the fractions from viscosity studies on the monomeric form, an average particle radius being calculated at about 36 Å. The polymers will, of course, be correspondingly

larger and thus more likely to yield thicker films if a comparison may be made with, say, γ -globulin. The results on reflected intensity for the β - and κ - forms are in accordance with the results obtained for the π -A curves of the two fractions (150). At constant area per molecule κ - casein shows much lower surface pressures than β , which indicates a thicker film for the κ - fraction, provided the surface densities are similar.

Ovalbumin

The notorious surface coagulating properties of ovalbumin make this protein unsuitable for a full study, but as a check on previous studies an experiment was conducted at pH 7.0 in 0.1 N KCl.

Results

Well cleaned surfaces brought together under 2 cm of hydrostatic pressure as quickly as possible (i.e. $\sim$ 2 minutes), yielded thin lamellae which were black to the eye. These were not stable for more than a few minutes, and were still thinning at a reflection of about 0.06% when bursting occurred. Ageing times of about 10 minutes produced more stable films with reflectivities around 0.2%. 20 minutes adsorption times gave films with 3 or 4 times this reflectivity. A photograph of a highly uneven film of ovalbumin is shown in fig 22, the areas of apparent even thickness being grey to the eye, as well as to the camera. All of the films

Free Film of Ovalbumin
(from 0.1% solution, long adsorption time)

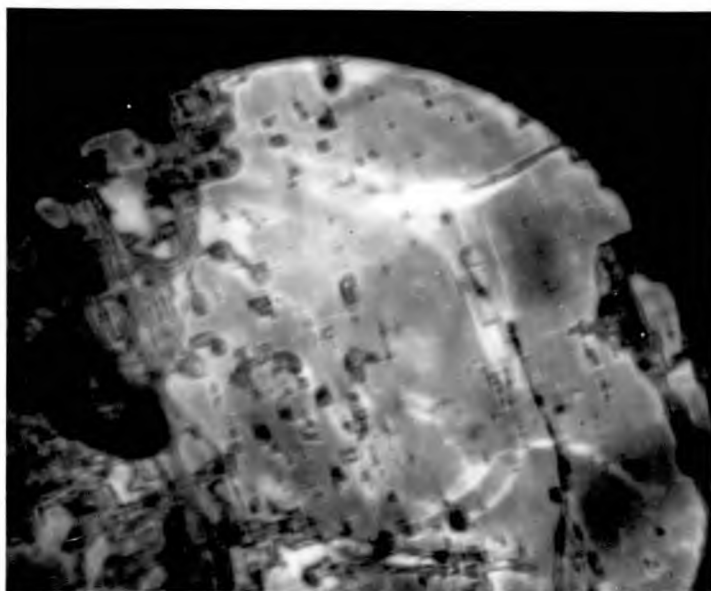


Figure 22

were capable of being stretched by increase in hydrostatic pressure to yield lower reflectivities.

Observations on bulk foams of protein

For all of the proteins studied, conditions which favoured persistent single lamellae also yielded "stable" foams ("stable" meaning that agitation of the solution yielded a head of foam of more than a centimeter in height, which persisted for minutes rather than seconds). Such conditions also produced large areas of film within the foam, even on gentle agitation e.g. BSA clearly demonstrates the 120° angle between surfaces which are often as large as 4 cm^2 in area. For such stable foams a hand lens may be used to observe interference colours. The lamellae were mobile on formation from fresh, filtered, solutions but settled to a more "rigid" behaviour after a few minutes, "rigid" as in the sense of Mysels (151) , often yielding parallel fringes. For the lamellae at the top of the foam, bursting usually took place on the occurrence of black regions. These formed at the top of the lamellae and also very close to the Plateau borders, and usually contained irregularities such as silver or yellow regions. For solutions foamed more than once, the picture was different. Irregular drainage was always obtained and black areas were found only infrequently. This behaviour has already been described for single lamellae and is probably caused by surface rearrangement which, in the limit, yields the visible coagulum observed for egg albumin, pepsin and hemoglobin. The

outstanding example of the stabilising effect of this coagulum is given by pepsin. This protein foamed only in water between pH 2 and 3.5 and it is this region where the "rigid" films are most easily formed.

Effect of molecular conformation on foam stability

Interesting behaviour is observed in solutions of protein in which disulphide bonds have been broken by urea and sulphite. It is only of interest to compare native and S—S reduced proteins for the cases of those with single polypeptide chains because competition for surface adsorption by multiple polypeptides renders interpretation difficult.

(i) 0.1 BSA solution in 8 M urea at 0.1 ionic strength foam to a much lesser extent than solutions in water. The same solution but with the ionic strength made up from sodium sulphite yielding a stable foam lasting longer than 0.1% BSA at 0.1 ionic strength pH 9.0.

(ii) 0.1% solutions of lysozyme at 0.1 ionic strength produced only small heads of foam until pH 10.5 was reached. 8 M urea produced a transient foaming solution but 8 M urea and 0.033 sodium sulphite yielded a foam stable for days. The foam often had an almost solid appearance and a very high surface gloss, completely different from normal foams from native proteins.

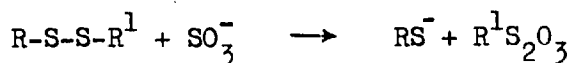
(iii) 0.05% solutions pepsin were caused to foam markedly outside the pH range 2.0 to 3.5 by the same procedure.

The viscosity change caused by the additions was not sufficient to cause the increases in apparent stability, as shown by the results in Table XX, which shows the times taken for solutions to flow a given distance down a capillary U-tube.

TABLE XX
Capillary flow times at 25°C

	<u>Water</u>	<u>8 M urea + NaCl</u>	<u>8 M urea + Na₂SO₃</u>
0.05% Pepsin	307 sec	384 sec	398 sec
0.1% BSA	305 sec	381 sec	390 sec

From such considerations it may be concluded that overall stability is not directly related to bulk viscosity in the cases considered. The changes in molecular configuration indicated by the flow times must be related to surface configuration and rheological properties before a full explanation is found. It is, however, obvious that the extra flexibility conferred on molecules by S—S reduction is conducive to foam stabilising properties. The possibility of repolymerisation via the S⁻ ions freed by the reaction;



should be absent since 0.03 M Na₂SO₃ is a molar excess.

Cumper (152) has shown that there is a relation between surface rheological parameters of the adsorbed proteins and the stability of protein foams. The work presented here supports this finding since surface configuration will have a direct bearing on the rheology.

Comparisons of foamed solutions of BSA, pepsin and lysozyme with and without S—S bonds are shown in figs 23, 24, 25.

Comparison of Foam of BSA + 0.09 MKCl and BSA +
Urea + Na₂SO₃ (0.03M) after 5 hours
(Constant Ionic Strength) pH 6.5



Figure 23

Comparison of Foam of Pepsin + 0.09 MKCl and
Pepsin + Urea + 0.03M Na_2SO_3 pH 6.5



Figure 24

Comparison of Lysozyme Foams pH 11.0



After 1 Hour



After 24 Hours

Left - Lysozyme
Centre - Lysozyme + 0.09 KCl
Right - Lysozyme + urea + 0.03 Na_2SO_3

Figure 25

APPENDIX I

Many proteins may be spread to yield a 'limiting area' of $1 \text{ m}^2 \text{ mg}^{-1}$. Consider BSA:

$$\begin{aligned} 1 \text{ m}^2 \text{ mg}^{-1} &= \frac{10^{20} \times 68,000}{10^{-3} \times 6 \times 10^{23}} \text{ \AA}^2 \text{ molecule}^{-1} \\ &= \underline{11,333 \text{ \AA}^2 \text{ molecule}^{-1}} \end{aligned}$$

The surface viscosity becomes appreciable at $0.8 \text{ m}^2 \text{ mg}^{-1}$ and this area is often used for a characteristic of a close packed surface film.

$\therefore$ A close packed film contains molecules occupying $9066 \text{ \AA}^2$. The molecular shape at 25°C , pH 5.15 has been estimated as a prolate ellipsoid of semi axes $69 \times 23 \text{ \AA}$ (53). If this is adsorbed in the native state to give a thickness at the surface of about $20 \text{ \AA}$ then;

$$\begin{aligned} \text{Cross section area} &= \pi ab \\ &= \pi \times 69 \times 23 \text{ \AA}^2 \\ &= \underline{4990 \text{ \AA}^2} \end{aligned}$$

Hydrodynamic data in 0.15N KCl (104) pH 7.4 yields axes of $65 \times 20 \text{ \AA}$.

$$\begin{aligned} \therefore \text{Cross section area} &= \pi \times 65 \times 20 \text{ \AA}^2 \\ &= \underline{4080 \text{ \AA}^2} \end{aligned}$$

The surface flattening of BSA is thus amply demonstrated.

APPENDIX II

04

DECK1 - EFN SOURCE STATEMENT - IFN(S) -

```

WRITE(6,103)
DC 50 N=1,140
1 READ(5,101) FN1,FN2,D1,D2,LAMBDA,FN,D,PHI
101 FORMAT(2F6.4,2F6.1,I5,F6.4,F6.1,F6.4)
PI=6.283186
FL=LAMBDA
B1=PI*FN1*D1/FL
B2=PI*FN2*D2/FL
R1=(FN1-1.0)/(FN1+1.0)
R2=(FN2-FN1)/(FN2+FN1)
A=2.0*(R1*SIN(2.0*B1+B2)-R1*R2*R2*SIN(2.0*B1-B2)+(1.0+R1*R1)*R2*
1 SIN(B2))
B=(1.0-R1*R1)*(COS(2.0*B1+B2)-R2*R2*COS(2.0*B1-B2))
C=(1.0+R1*R1)*(SIN(2.0*B1+B2)-R2*R2*SIN(2.0*B1-B2))+4.0*R1*R2*
1 SIN(B2)
FJ1=(A*A)/(B*B+C*C)
FJ2=(PI*FN*D*COS(PHI))/FL
FJ2=((FN-1.0)/(FN+1.0))*SIN(FJ2)
FJ2=FJ2*FJ2*4.0
50 WRITE(6,102)FN1,FN2,D1,D2,LAMBDA,FJ1,FN,D,LAMBDA,PHI,FJ2
102 FORMAT(5X,2(F6.4,5X),2(F6.1,5X),I5,5X,F8.5,10X,F6.4,5X,F6.1,5X,I5,
15X,F6.4,5X,F8.5)
103 FORMAT(1H1,10X,25HREFLECTANCE OF THIN FILMS,///
16X,3HFN1, 8X,3HFN2,9X,2HD1,9X,2HD2,6X,6HLAMBDA,7X,3HFJ1,15X,
12HFN,9X,1HD,7X,6HLAMBDA,6X,3HPhi,8X,3HFJ2)
104 STOP
END

```

SPECIMEN COMPUTER PROGRAMME

APPENDIX III

The Langmuir Method of Proving Irreversibility of Protein Adsorption as Applied to B.S.A.

Consider the Gibbs equation:

$$\frac{dF}{d(\ln c)} = \sigma kT$$

where σ = surface concⁿ in
molecules cm⁻²

F = surface pressure

c = bulk concentration

$$d(\ln c) = \frac{dF}{\sigma kT}$$

$$\ln c_1 - \ln c_0 = \frac{F_1 - F_0}{\sigma kT}$$

$$\log_{10} \frac{c_1}{c_0} = \frac{F - F_0}{2.303 kT \sigma}$$

Consider the increase in solubility for $F - F_0 = 1 \text{ dyne cm}^{-1}$

Put $S = \log_{10} c_1/c_0$

$$S = \frac{1}{2.303 \times 1.37 \times 10^{-16} \times 298 \times \sigma}$$

$$\text{At } 1 \text{ m}^2 \text{ mg}^{-1} \quad \sigma = \frac{10^{-3} \times 6 \times 10^{+23}}{68,000 \times 100 \times 100} \quad \frac{\text{molecules}}{\text{cm}^2}$$

Assuming a molecular weight of 68,000

Appendix III continued

$$S = \frac{68 \times 10^7}{2.303 \times 1.37 \times 10^{-16} \times 298 \times 6 \times 10^{+20}}$$

$$\underline{S = 12}$$

∴ Increase in solubility = 10^{12} fold.

No such increase is observed and thus the Gibbs equation for reversible adsorption is not applicable.

SUMMARY OF CONCLUSIONS

1. The limiting thickness of free films formed from BSA solutions containing enough KCl to depress the electrical double layers was not found to be equal to either twice any native molecular dimension, nor to twice the estimated thickness of a spread BSA molecule. The molecular reorganisation of BSA on adsorption at the air-water interface has thus been shown not to be as simple as often reported in the literature. Electrostatic repulsion does not control the limiting thickness (as in soap films); the final stage of thinning is controlled by the rheological properties of the surface layers.
2. The molecular reorganisation is consistent with an unravelling ('denaturation') of the protein molecule since surface activity requires the molecule to present hydrophobic regions to the non-aqueous phase and hydrophilic regions to the aqueous phase. The unravelling is more prevalent in the molecules which arrive first at the interface. Molecules arriving later are unable to unravel fully against the surface pressure caused by those preceding them. As the surfaces are stretched by flattening, the less unravelled molecules 'denature' further to take up the extra surface area.
3. Protein adsorption is irreversible if denaturation is complete because proteins contain hydrophobic amino acid residues which 'anchor' the molecules to the surface at different points along

the polypeptide backbone and all of these must be removed simultaneously before the molecule can pass back into solution. Thus most adsorbed protein molecules are irreversibly adsorbed from solution but there is a possibility that those arriving during the last stages of surface coverage are reversibly adsorbed.

4. The other proteins studied (bovine gamma globulin, pepsin, lysozyme, insulin, the caseins and ovalbumin) showed similar characteristics but absolute analysis of the results was complicated by the lack of information on the necessary parameters. All of the proteins studied were capable of producing 'black' films providing small adsorption times were allowed. These films were, however, transient in nature and only BSA produced stable uniform black films after long adsorption times. This was probably because BSA film surfaces could be cleaned and conditions controlled so that relatively quiescent systems were studied. Under these conditions BSA was the least prone to ejection of parts of the polypeptide backbone from the interfacial region and the ultimate production of 'surface coagulum'. This is defined as the three dimensional matter produced at interfaces by localised high surface pressures and caused by surface agitation. All of the proteins which yielded stable films were capable of producing surface coagulum if the surfaces were agitated enough.

5. Removal of intra-molecular constraints in protein molecules allows free films and foams to be formed which are more stable than those obtained from the native molecules. Molecular orientation at interfaces has been used to describe the increase in thickness of surface films and also the increased stability. In general, solutions which produced stable foams also yielded stable lamellae. The most persistent foams and films were characterised by inhomogeneous surface layers containing surface coagulum.
6. An attempt to find a surface chemical model for proteins among commercially available synthetic polypeptides failed because proteins contain a delicate balance of hydrophilic and lipophilic properties combined with aqueous solubility. This is not matched by copolymers since the lipophilic groups necessary for surface activity cannot be separated by enough hydrophilic groups to confer solubility without destroying the surfactant properties. This is not surprising since proteins contain some twenty different amino acids distributed in a molecule of some hundreds of amino acid residues.

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