The Behaviour of Proteins at the Liquid-Liquid Interface

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An interesting recent development in the study of unimolecular films has been the employment of such films in the determination of the molecular weights of proteins. Guastalla (1939, 1943), working with his sensitive floating-thread surface balance, made the surprising discovery that unimolecular films of proteins at the surface of water behaved in the manner of non-ideal two-dimensional gases at low surface pressures. He was consequently able, by application of the gas laws, to estimate the molecular weights of a number of proteins in the surface phase.

The equation of state for an ideal two-dimensional gas may be written:

$$FA = nRT, \quad (1)$$

where F represents the surface pressure in dynes/cm., A the area of the gas in sq.cm., n the number of moles of gas, T the absolute temperature and R the two-dimensional gas constant with a value of 8.31×10^7 ergs/mole/degree (cf. Adam, 1941).

For non-ideal surface gases, Volmer & Mahnert (1925) suggested a formal analogy with the van der Waals equation for real gases:

$$(F + a/A^2)(A - b) = RT, \quad (2)$$

where a is a constant determined by the forces of attraction between the molecules in the surface and b is a constant proportional to the area occupied by the molecules in the surface.

The plots of FA against F obtained by Rideal & Schofield (1925, 1926) for adsorbed films of the lower fatty acids, and by Adam & Jessop (1926) for insoluble films of certain long-chain dibasic esters at the water surface, have the characteristics required by such an equation of state, namely a minimum related to the a/A^2 term and an intercept on the FA -axis approximating to the theoretical value of RT . There appears to be little doubt that an equation of the van der Waals type will describe the behaviour of a gaseous surface film, at least in the first approximation.

Guastalla (1939, 1943), for his protein films, plotted the quantity C/F against C , where C represented the surface concentration in mg. protein/sq.m. The curves thus obtained showed a linear portion at low concentrations which could be

extrapolated to $C = 0$. The intercept on the C/F axis gave a value for $1/nRT$ from which the molecular weight of the protein could be calculated. The values found for gliadin (27,000) and egg albumin (40,000) agreed well with the accepted values from osmotic pressure measurements in bulk phase. On the substrate employed (0.01N-HCl) haemoglobin apparently underwent dissociation into fragments of mean molecular weight 12,000.

Bull (1945, 1946, 1947, 1950) and Dieu & Bull (1949) have reported an extensive series of experiments on films of proteins at the air-water interface, mainly carried out with a surface balance of the Wilhelmy type. They find that all such films, with the exception of zein, give $FA-F$ plots in which no minima occur. In many cases the relation is quite rectilinear, suggesting an absence of appreciable interaction between the protein molecules, and extrapolation to $F = 0$ may be performed with ease. Bull gives the equation for his straight-line graphs in the empirical form:

$$FA = \alpha F + \beta, \quad (3)$$

where α represents the slope of the line and β the intercept on the FA -axis. With the assumption of the validity of the ideal gas laws at zero surface pressure, β will then give the value of nRT and the molecular weight of the molecules constituting the film may be calculated. When F is expressed in dynes/cm. and A in sq.m./mg. protein, it is clear that at 25°:

$$\beta = 8.31 \times 298 \times 10^7 \times n \text{ ergs,}$$

whence the molecular weight

$$M = \frac{8.31 \times 298}{\beta} = \frac{2460}{\beta}.$$

The slope α of the line will give the area in sq.m./mg. occupied by the protein molecules in the uncompressed state.

In the most recent of his papers, Bull (1950) gives a thermodynamic derivation of Eqn. 3 in which the constants prove to have the meaning previously attributed to them. This derivation tacitly assumes an absence of interaction between the molecules constituting the surface layer, an approximation that appears to be justified by the experimental results.

On the basis of these considerations, Bull has found molecular weights for egg albumin, bovine

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serum albumin, insulin, rabbit γ -globulin, pepsin, trypsin, chymotrypsinogen and trypsinogen, all spread on ammonium sulphate solutions, corresponding within the limits of experimental error to values previously established by osmotic pressure measurements and with the ultracentrifuge. Zein, to which Bull (1947) only alludes in passing, is peculiar in that the $FA-F$ curve shows a minimum and the molecular weight (20,100) shows dissociation to have taken place on the surface. β -Lactoglobulin shows half the bulk-phase molecular weight (17,100) on ammonium sulphate. When, however, copper ions are present in the substrate, a value showing no dissociation (34,300) is found.

The data at present available concerning the behaviour of protein films at the interface between two liquids (Danielli, 1938; Askew & Danielli, 1940; Alexander & Teorell, 1939; Cheesman, 1946a; Cumper & Alexander, 1950) indicate that at areas above 1 sq.m./mg. a film of a given protein exerts under such conditions considerably higher pressures than does a film of the same protein at the air-water interface. It would seem that no investigation has been made into the state of the interfacial protein films at large areas, although Cumper & Alexander (1950) have referred to them as being of the vapour-expanded type. The findings of Bull, however, demonstrate clearly the gaseous nature of protein films at the air-water interface, often at areas as low as 1.5 sq.m./mg. Since the pressures exerted by corresponding films at the liquid-liquid interface at similar areas are considerably higher, it is difficult to conceive that these are not also in the gaseous state. The differences in the pressure-area isotherms for the two types of film at low pressures could be accounted for by different states of association of the molecules, different orientations of the molecules in the interface, or a combination of these and other factors. It was in an endeavour to elucidate this matter that the present investigation was made.

EXPERIMENTAL

Apparatus. In view of the fact that the author (Cheesman, 1946a) had already applied the Wilhelmy hanging-plate technique with some success to the study of protein films at the liquid-liquid interface, it was decided to attempt its adaptation to the present end. The apparatus is shown diagrammatically in Fig. 1. The trough was a cylindrical glass dish of about 12 cm. diam. and 3 cm. depth. It was accommodated in an adapted copper water oven which in turn was mounted in a stout wooden box. On the top of this rested a sensitive analytical balance. To the outer jacket of the oven were soldered inlet and outlet tubes to permit a constant flow of water pumped from a thermostat bath. Two tubes of 3 mm. diam. were soldered through the jacket. Through one of these passed wires from an external transformer to provide current for a flash-lamp bulb mounted on the roof of the oven. The other was arranged to correspond with a hole in the base of the balance case. Through this hole

and tube passed a silk thread terminating at its upper end on a hook on the left-hand balance pan and at its lower end in a small wire hook on which the hanging plate could be suspended. A thick Perspex window was fitted in the door of the oven and in this was made a hole, oblique in the downward sense, to fit the barrel of an 'Agla' micrometer syringe. This hole was normally closed with a rubber bung. A thermometer, reading to 0.1°, stood inside the oven. A wad of

Fig. 1. Diagram of the interfacial film balance.

cotton wool, saturated with the non-aqueous phase of the two-phase system, was placed in the oven during experiments. This served to saturate the gaseous phase with the vapour and to minimize the error due to evaporation from the trough. The hanging plate was a microscope cover-slip ($4 \times 3 \times 0.015$ cm.), to the upper corners of which a loop of silk thread was attached by sealing wax. The beam of the balance carried at its centre a concave mirror. An optical lever of 150 cm. length magnified the movements of the beam.

Procedure. The balance was calibrated by adding weights to the right-hand pan when the plate was hanging in a clean water surface. From the data thus obtained and the dimensions of the slide, the factor was calculated for converting the readings of the light spot into dynes/cm. The sensitivity of the balance was such that a change in surface tension of 1 dyne/cm. resulted in the deflexion of the light spot by about 3 cm. It was thus possible to measure surface pressures with an error of somewhat less than ± 0.01 dyne/cm.

An experiment was performed in the following manner. The glass dish was cleaned in hot chromic-sulphuric acid and washed with dilute phosphoric acid and distilled water. It was then transferred to the copper oven with crucible tongs. The aqueous phase was added to a depth of 1 cm. and the water surface was cleaned by suction. When benzene was used as oil phase, this cleaning process could be supplemented by using a drop of benzene as a piston to force contaminants to the side of the dish (Alexander & Teorell, 1939). A layer of oil phase of about 1 cm. thickness was then poured into the trough. Both liquids had been stored in the water thermostat so that undue time might not elapse in the attainment of temperature equilibrium. The glass plate was cleaned in its effective region with hot chromic-sulphuric

acid, washed with phosphoric acid and distilled water and dried in a blast of hot filtered air. It was then connected to its suspension and placed in position in the oil-water interface. The door of the oven was closed and the equilibration allowed to proceed until the position of the light spot was constant over 5 min. During the equilibration the sensitivity of the apparatus was controlled by the addition of weights to the right-hand balance pan. If the sensitivity proved to be identical with that when a clean water surface was employed, it could be assumed that the contact angle between the glass and the water at the oil-water interface was zero. If this did not prove to be the case, the plate was contaminated and it was necessary to recommence the experiment.

Protein was spread from 0.05% (w/v) solution in 60% (v/v) isopropanol, 0.5M with respect to sodium acetate. The spreading of protein from such solvents has been shown to be rapid and complete, even against considerable surface pressures, at both the air-water and benzene-water interfaces (Stållberg & Teorell, 1939; Alexander & Teorell, 1939).

Additions of protein to the interface were made by passing the micrometer syringe through the hole in the Perspex window and, with the needle tip in the oil phase, applying drops of the solution to the water surface. After each such addition of about 2 μ l. an interval of 2 min. was allowed to elapse before a surface pressure reading was taken. This allowed for the dispersion of the interfacial accumulation of propanol which was found in blank experiments to have no sensible effect upon the results when this precaution was observed. About twenty surface pressure readings were made over the range 0–1 dyne/cm. The corresponding areas were calculated from the amount of protein spread and the cross-sectional area of the trough.

Materials and methods. All inorganic reagents were of analytical quality and free from surface-active impurities. The water employed was distilled water from the laboratory supply redistilled from alkaline permanganate in a Pyrex apparatus. The benzene and cyclohexane were of analytical grade and redistilled in a Pyrex apparatus.

The bovine serum albumin was a crystallized specimen supplied by Armour and Co. A specimen of pure human carboxyhaemoglobin was kindly presented to the author by Dr B. Cinader of the Lister Institute.

The albumin was weighed out after drying in a thin layer for 24 hr. *in vacuo* over H_2SO_4 . The carboxyhaemoglobin was provided in 4.0% (w/v) solution in water. The concentration was confirmed by surface film measurements in the Langmuir trough (cf. Jonxis, 1939).

RESULTS

Human methaemoglobin at the benzene-water interface

Carboxyhaemoglobin dissolved in the propanol-sodium acetate mixture undergoes rapid conversion to methaemoglobin in the presence of air. That denaturation does not take place is evidenced by the fact that it is an easy matter to reconstitute the carboxyhaemoglobin by reduction with sodium hydrosulphite and subsequent passage of carbon monoxide. The changes in the prosthetic group are readily followed spectroscopically.

The points for six determinations on methaemoglobin at the interface between benzene and 0.001M-sodium phosphate buffer (pH 6.8) at 25° are shown in Fig. 2. The full-drawn curve is drawn through the points representing the arithmetic means at ten arbitrary pressures. The $FA-F$ curve appears to consist of two rectilinear sections, with a change of slope at about 0.4 dyne/cm. Extrapolation of the lower portion to $F=0$ gives $\beta=0.24$ and the molecular weight $M=10,200$. The disposition of the points suggests, however, that the linear relationship does not hold at pressures below

Fig. 2. $FA-F$ curve for human methaemoglobin at the interface between benzene and 0.001M-sodium phosphate buffer, pH 6.8, at 25°.

0.1 dyne/cm. A more probable extrapolation gives $\beta=0.28-0.32$, and $M=8200 \pm 400$. The slope of the lower portion of the curve, α in Eqn. 3, gives 1.33 sq.m./mg. for the area occupied by the protein in the uncompressed state. The upper portion of the curve gives $\alpha=1.16$ sq.m./mg. It seems probable that the change of slope at 0.4 dyne/cm. marks a transition between two types of orientation of the molecules in the surface.

Bovine serum albumin at the benzene-water interface

The results obtained with six films of bovine serum albumin at the interface between benzene and 0.001M-sodium phosphate buffer (pH 6.8) at 25° are shown in the $FA-F$ plot in Fig. 3. The full-drawn curve represents the arithmetic mean of the individual curves calculated for nine arbitrary pressures. The presence of a minimum appears to be well established at a pressure (0.2 dyne/cm.) corresponding to that observed by Bull (1947) with zein films at the air-water interface. Extrapolation

of the curve to zero pressure is rendered difficult by its form, but it seems probable that the value for β lies in the range 0.52–0.60, which gives an apparent molecular weight of 4400 ± 300 . It is interesting to adduce Bull's comment to his zein curves that the minimum might be due not to attractive forces between the molecules in the film, but rather to the expulsion with increasing surface pressure into the aqueous phase of low-molecular material. There would seem at present to be no means of deciding between these alternatives. Analogous determinations at the air-water interface, described below, gave no evidence of a minimum and it seems hardly probable that impurities in the serum albumin can have been responsible for the effect.

Fig. 3. FA - F curve for bovine serum albumin at the interface between benzene and 0.001 M-sodium phosphate buffer, pH 6.8, at 25° .

The slope of the rectilinear part of the curve gives a value of 0.75 sq.m./mg. for the area occupied by the protein in the uncompressed state.

Bovine serum albumin at the air-water interface

The apparent extensive dissociation of bovine serum albumin at the benzene-water interface made it desirable to investigate whether the protein spread from propanol solution would exhibit its native molecular size at the air-water interface. Appropriate experiments were therefore carried out with the same technique. Difficulty was found, however, in maintaining a zero contact angle between the surface and the hanging plate in the low-pressure region. Thus, although it was found that the apparent molecular weight was much higher under these conditions, it was thought unjustified to make an exact calculation. Bull (1950), however, en-

countered similar difficulties with egg albumin films and found that the contact angle righted itself at pressures above 0.5 dyne/cm., so that an extrapolation of the rectilinear part of the FA - F curve gave a reasonable value for the molecular weight. An examination of our results in the light of Bull's findings showed that the FA - F plot was, in fact, quite rectilinear at pressures above 0.15 dyne/cm. and gave a molecular weight value of $70,000 \pm 3000$. As this corresponds closely with the osmotic pressure value for the protein (Adair & Adair, 1930), the serum albumin evidently does not undergo irreversible dissociation in propanol solution. The slope of the curve gave a value for α of 0.81 sq.m./mg., a figure much lower than that recorded by Bull (1950) for the same protein spread from aqueous solution on 5% (w/v) ammonium sulphate (1.2 sq.m./mg.).

Protein films at the cyclohexane-water interface

The interfacial tension between cyclohexane and water (50 dynes/cm.) is higher than that at the benzene-water interface (35 dynes/cm.). In an attempt to determine whether the interfacial energy would influence the behaviour of a gaseous protein film, a large number of experiments were made in which the proteins were spread at the interface between phosphate buffer and cyclohexane. The FA - F curves for bovine serum albumin and human methaemoglobin often corresponded within the limits of experimental error with those obtained at the benzene-water interface. Reproducibility, however, was not good, and the behaviour of the methaemoglobin films was especially erratic, values as low as 3000 sometimes being obtained for the molecular weight. No adequate explanation could be found for this. Every effort was made to eliminate contamination. Lability of the contact angle may have been a contributing factor, although this was not visually evident. Whether methaemoglobin does, in fact, undergo extensive dissociation at the cyclohexane-water interface, under the influence of an unknown and hitherto unreproducible factor, must remain for the present an open question.

Films of heat-denatured proteins at the benzene-water interface

It has been shown by Stållberg (1939) that serum albumin in propanol-sodium acetate solution will undergo true heat-denaturation without simultaneously being precipitated. This has been confirmed for a number of proteins by the present author (Cheesman, 1946c; unpublished observations). It was clearly of interest to study the effect of such denaturation on films spread under the conditions of the present work. Bovine serum albumin and human methaemoglobin were accordingly spread at the benzene-phosphate buffer interface from solutions

previously heated to 90° for 10 min. Within the limits of experimental error, no significant differences were found between these and films spread from unheated solution.

DISCUSSION

On the assumption that a protein film, at infinite expansion, may be treated as a perfect gas, the above results suggest that protein molecules undergo dissociation at the oil-water interface into fragments of a surface-active nature. If we assume values of 66,000 and 70,000 for the molecular weights of human methaemoglobin and bovine serum albumin respectively, these fragments would seem to have a mean weight in the former case of about one-eighth and in the latter about one-sixteenth of that of the undissociated molecule at pH 6.8.

The author (Cheesman, 1946*b*) has previously found indications that unimolecular films of protein are oriented in different manners at the air-water and the oil-water interfaces, and this has recently been established by Davies (1951) in his ingenious measurements of the electrical phase boundary potentials of protein films at the petroleum ether-water interface. It may be that a more rigid orientation of non-polar side chains towards the oil brings about a disruption of secondary intramolecular linkages which remain unaffected at the air-water interface.

It is tempting to suggest a relation of the present finding with the now discounted hypothesis of Sørensen (1930) according to which the serum proteins, and many others, are systems of sub-

molecular components linked by residual valencies and capable of reversible dissociation.

An interfacial dissociation of native proteins could clearly be of interest in relation to proteogenesis. There is little doubt that the biological synthesis of proteins takes place at phase boundaries within the cell. If protein molecules have a structure of sub-units in physical association, the question presents itself whether the final stage of the synthesis may be regarded as a specific adsorptive organization into a single entity of a number of enzymically synthesized precursors.

SUMMARY

1. A method has been devised for the study in the low-pressure region of unimolecular films of proteins at the interface between two liquids.

2. Films of human methaemoglobin and bovine serum albumin have been studied at the benzene-water interface.

3. The laws for a two-dimensional gas have been applied to the experimental data. It is concluded that the molecule of human methaemoglobin dissociates into about eight, and that of bovine serum albumin into about sixteen kinetic units when adsorbed at the benzene-water interface at pH 6.8.

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