LXXI. THE INFLUENCE OF THE ELECTROLYTE CONTENT OF THE MEDIUM ON THE PROPERTIES OF COMPLETELY SPREAD PROTEIN FILMS

By GEORG THEODOR PHILIPPI

From the Institute of Physical Chemistry, University of Upsala and the University of Leyden

(Received 12 September 1936)

INTRODUCTION

FOR the investigation of protein films at air/water interfaces two methods have been used:

- (a) Determination of the drop in surface tension which the layers bring about.
- (b) Determination of the surface potential change which the layers produce.

The apparatus used resembles somewhat the spreading apparatus described earlier in the literature. In several respects, however, the apparatus has been improved, e.g. in the use of a new type of differential balance for force-area determinations. A full description has already been given elsewhere [Philippi, 1936].

MODE OF PREPARATION OF THE PROTEINS USED

The proteins used were kindly put at my disposal by several investigators and were prepared as follows:

Egg albumin was prepared by Dr J. van Ormondt according to Sørensen's method. The protein was recrystallized 5 to 6 times and then dialysed against distilled water until the sulphate reaction of the water remained negative after standing 48 hours in the dialyser. The concentration of the solutions was determined by the Ter Meulen-Heslinga method [1930], assuming a nitrogen content of 15.1 % [Calvery, 1931].

During the author's stay in Prof. Svedberg's laboratory a sample of such an egg albumin solution was tested in the ultracentrifuge. The preparation was quite homogeneous and the sedimentation constant of the protein $S_{w,20} = 3.53 \cdot 10^{-13}$ was close to the value found earlier in Svedberg's Laboratory.

The *insulin* studied was Allen and Hanbury's brand. The solutions supplied by the manufacturers contained 80 units per ml. The protein was recrystallized according to the method of Scott [1931] by van Ormondt. After dialysis against dilute hydrochloric acid, pH 2.5, concentration determinations of the solutions were carried out according to the Ter Meulen-Heslinga method, assuming a nitrogen content of 14 % [Scott, 1925].

A series of experiments was also carried out with the unpurified material, containing 80 units per ml. The results were very much the same as with the recrystallized material, but the surface areas were somewhat smaller. This may be due to an error in the concentration determination of these non-dialysed solutions.

Pepsin. A sample of pepsin (Northrop), dissolved in glycerol, was given me by Prof. E. Gorter. The concentration of this stock solution was 5 %. Before spreading it was diluted 10-fold with an acetate buffer of pH 4. At this pH self-digestion of the proteolytic enzyme is much reduced.

A sample of pepsin (Philpot) was given me by Dr Philpot, prepared as previously described [1935]. Before spreading, this pepsin also was dissolved in an acetate buffer of pH 4. Concentration determinations were carried out by Mr K. Andersson by Kjeldahl's method.

Lactoglobulin Palmer. This protein was given me by Prof. Svedberg. For details as to the preparations see Palmer [1934] and Pedersen [1936]. The solutions used were prepared by dissolving a known quantity of the protein, which had been dried over P_2O_5 in a known volume of 0.02N acetate buffer containing 0.04N KCL.

Serum albumin was given me by Miss Brosteaux. The protein was prepared according to Svedberg & Sjögren [1928]. The preparation was tested in the ultra-centrifuge and appeared to be quite homogeneous. The sedimentation constant was $S_{W, 20} = 4.61 \cdot 10^{-13}$.

The concentration of the serum albumin solution was determined by dry weight measurements both on the solution and on the solvent.

EXPERIMENTAL RESULTS AND THEIR INTERPRETATION

Recently it has been pointed out [Philippi, 1936] that the area A_0 of protein films, found by extrapolation of the linear part of the force-area curves to zero force, is independent of the pH of the medium. This result does not, however, imply that no changes take place as the pH varies.

When the films are compressed to an area which is not less than the area A_0 , remarkable changes of the film area (at constant pressure) are observed. An example of such changes is shown in Fig. 1.

Fig. 1.

The surface potential difference, ΔV , also changes very much with pH. For the s.p.d. area¹ curves are shifted nearly parallel to themselves the shift being somewhat larger at smaller areas (Figs. 2, 3 and 4). To a first approximation s.p.d. area curves are also shifted parallel to themselves if the salt content of the solution is changed. The influence of KCl is shown in Fig. 3.

In Fig. 4 the ΔV values for an area of 0.75 m²/mg. are plotted as a function of pH for several proteins.

¹ Surface potential difference will be represented by s.p.d.

Fig. 2.

Fig. 3.

Fig. 4.

The determinations for the upper egg albumin curve were done on 0.0033*N* acetate buffers, those for the lower curve on 0.02*N* acetate buffers.

Insulin was spread on veronal buffers containing 0.033*N* sodium acetate, 0.0033*N* sodium veronal and a varying amount of HCl.

Both pepsin preparations were spread on veronal buffers of the same concentration, but the solutions contained 0.01*N* KCl as well.

The difference between the two egg albumin curves in Fig. 4 shows the influence of the sodium ion on the surface potential values. In general, the slope of

ΔV - pH curves is decreased by increasing salt content. Preliminary experiments with salts of different valencies revealed that the slope $d\Delta V/d\ pH$ diminishes to zero value when the ionic strength of the medium is sufficiently high.

In Fig. 1 the variation of the area of lactoglobulin films, at constant pressure, has been plotted as a function of pH . Interesting curves are obtained also by plotting the film pressure as a function of pH at constant area. Examples are shown in Figs. 5 and 6.

To explain both surface potential and film pressure changes it must be decided whether these changes are due to reorientation of the polypeptide molecules, to changes in their ionization, or to both combined.

Recently it has been shown [Philippi, 1936] that no reorientation of the molecules occurs unless the medium becomes very acid ($pH < 2$). Moreover, it has been shown that the film pressure variations, at constant area, are due to the following factors:

- (a) The electric repulsion between equally charged film molecules or parts of these molecules.
- (b) The electric repulsion between equally charged counter-ions.
- (c) The osmotic forces exerted by the counter-ions. These ions are at an excess concentration in the neighbourhood of the surface.
- (d) An occasional penetration of counter-ions and other substances between the polar heads of the molecules. Such a penetration may be due either to ordinary electrostatic attraction or to a specific adsorption.

To a first approximation the film pressure variations ΔF are fixed by the following equation:

$$\Delta F = B\sqrt{c} \left[\sinh \frac{L\psi}{4RT} \right]^2, \quad \dots\dots(1)$$

where ΔF = the difference between the film pressures of a charged and a neutral film of equal area,

ψ = the difference of the surface potential of such films,

c = the ionic concentration in the bulk of the solution,

L = the charge of one gram equivalent in E.S.U.,

R = the gas constant,

T = the absolute temperature,

B = a known constant.

In the right-hand side of equation (1) ψ can be calculated from surface potential measurements, whereas all other factors are known constants.

Hence it is possible to calculate film pressure variations from the corresponding surface potential variations without making use of any assumptions other than those involved in formula (1).

Such a calculation has been carried out using data derived from experiments with insulin. The theoretical values of the film pressure variation ΔF have been used to construct the full line curve of Fig. 7.

In the same figure the experimentally observed ΔF values, measured at a constant area of 1.40 m²/mg., have been indicated by points. For the calculation of ΔF the minimum pressure in the F - pH curve, situated at about pH 5.4, has been used as reference pressure. The experimental points are connected by a dotted line.

Fig. 7 shows several interesting features. First there is, within the limits of experimental error, a coincidence of the minimum of the experimental F - pH curve and the isoelectric point of insulin, which is at about pH 5 according to measurements by Wintersteiner & Abramson [1933]. The experimental F - pH curve of lactoglobulin (Fig. 1) shows the same feature, the isoelectric point of

lactoglobulin being $pH\ 5.19$, according to Pedersen. Hence it appears that the isoelectric points of a protein in the dissolved state and in the spread state are the same within the limits of experimental error. If there is any difference at all, it can only be a small one. This fact definitely shows that the observed film

Fig. 5.

Fig. 6.

Fig. 7.

pressure variations are at least partly electrical in origin, and moreover the agreement between the theoretical curve and the experimentally observed values in Fig. 7 in the pH range 2–6 shows that the film pressure variations are brought about by the factors which are taken into account in equation (1), viz. electric repulsions and osmotic forces.

However, both at $pH < 2$ and $pH > 6$ the experimental and theoretical pressure values disagree. As has been shown earlier [1936, p. 83], the deviation below $pH 2$ is due to the fact that the assumptions made in equation (1) are no longer valid if the ionic strength of the medium becomes high. The deviation at $pH > 6$ is due to association of the molecules in the surface.

In Figs. 5 and 6 the pressures of both serum albumin and insulin films hardly changes with pH in the range which includes the isoelectric points of these proteins. Obviously, no film pressure variation can be measured in the extreme case, when all molecules are associated and form a quite rigid surface layer. This may be illustrated by the following example: At a mercury-water interface considerable changes of the interfacial tension and area can be measured when the electric charge of the mercury surface is changed, the action of the well-known capillary electrometer being due to such changes. At solid metal-water interfaces no such phenomena are observed. The electric and osmotic forces, however, are just as much present as in the mercury-water interface, but owing to the rigidity of the solid surface of the metal they cannot bring about any measurable change either in surface tension or in surface area. The independence of the pressure of some protein films of their charge is due to the same cause, i.e. the rigidity of the surface layer due to association of the spread molecules.

The deviation above $pH 6$ between the experimental and theoretical curves in Fig. 7 is also due to association of the spread insulin molecules.

pH STABILITY REGIONS OF THE FILM AREA AND OF THE FILM PRESSURE

Some proteins do not spread out over the whole available area of the water surface, but form a coherent film. The areas of such films can easily be measured with the movable air electrode [Philippi, 1936]. The properties of coherent films and the possibility of their formation depend on the pH of the medium in every case in which the phenomenon has been observed. An example is shown in the upper part of Fig. 6. When the pH of the solution, on which the insulin is spread, is near to the isoelectric point of the protein, a coherent film forms, the area being minimum in this pH range.

Although conclusions as to the isoelectric point of the films can easily be drawn from curves of the type shown in Figs. 1 and 5, no such conclusion can be drawn from Fig. 6, as the observed areas of these coherent films are not very reproducible. The peculiar form of the lower curve in Fig. 6 will now be clear also. The pressures were read at a constant area of $1.75 \text{ m}^2/\text{mg}$. As this area is somewhat larger than the minimum value for the area of the coherent film, the film pressures fall to zero in a narrow pH range near the isoelectric point. Apart from this narrow range the film pressure is almost independent of the hydrogen ion content of the medium at $pH > 4$.

A similar independence of the film pressure is observed with serum albumin films (Fig. 5). The phenomenon indicates that in serum albumin films small coherent islands exist, although no macroscopic "coherent film" could be detected with the air electrode. The resolving power of this "film microscope" appears to be too small to detect the islands in the serum albumin films.

Egg albumin and pepsin, however, form macroscopic coherent films. The areas of these films scarcely vary with the pH of the medium over a rather wide pH range in the neighbourhood of the respective isoelectric points of these proteins. The phenomenon is shown in Fig. 8 and in Table I.

Table I. *Pepsin (Philpot) spread on HCl and on veronal buffers containing 0.0033N sodium acetate, 0.0033N sodium veronal, 0.01N KCl and a varying amount of HCl*

Area coherent film in m ² /mg.	pH	Buffer
1.00	1.98	HCl 0.01 N, KCl 0.01 N
1.08	2.80	Veronal buffer
1.03	3.59	—
0.82	4.36	—
0.39	4.95	—
∞	7.11	—
∞	7.58	—
∞	8.12	—

In the pH range 4–7 pepsin does not spread completely on buffer solutions of the ionic strength used.

Fig. 8.

Fig. 9.

Both in the case of egg albumin and of pepsin, it is possible to define a pH stability region of the film area. Outside that pH range the cohesion in the films is much less and they expand owing to electric and osmotic forces, which act along the surface.

Serum albumin and insulin films also show a similar pH stability region. By plotting the film areas as a function of pH, at constant pressure, this is easily demonstrated. An example of a pH stability region found in this way is shown in Fig. 9.

Similarly pH stability regions of the film pressure, at constant area, exist. As a matter of fact they have been shown already in Figs. 5 and 6.

The relations between these pH stability ranges of protein films and the pH stability ranges of dissolved protein molecules found by Svedberg and his collaborators have been discussed recently [Philippi, 1936].

Not all protein films possess a "pH stability region of the film area". Lactoglobulin films, for instance, do not, as can be seen from Fig. 1. In this respect differences between the various proteins are due to a different surface solubility of their molecules. When the surface solubility of spread molecules is zero, all molecules are associated (in two dimensions) into a coherent film. If the surface solubility is larger, only a part of the molecules is associated into small or large coherent islands, the other part being dissolved in two dimensions. The experiments with insulin show that the tendency to form a coherent film is a maximum

at the isoelectric point of the protein. Hence the surface solubility of insulin is a minimum at its isoelectric point.

With the other proteins investigated no minimum area of the macroscopic coherent film at their respective isoelectric points was found. In these cases the surface solubility must be constant over a pH range near the isoelectric point.

SUMMARY

1. The surface potential difference ΔV produced by protein films depends markedly upon the pH and the salt content of the medium. To a first approximation, variations of the potential drop across the films are independent of their states of compression.

2. For sufficiently large areas (e.g. within the range of 1.0–2.0 m²/mg.) the film pressure of a protein film, with reference to a constant area, depends markedly on the pH of the medium.

These changes of the film pressure are due partly to electric forces between the film molecules and between the counter-ions, and partly to osmotic forces brought about by the excess concentration of the counter-ions close to the surface. In some cases ionic penetration likewise increases the film pressure.

3. It was found possible to calculate, from the corresponding variations of the surface potential, the variation of the film pressures of spread protein films (with reference to a constant area).

4. In the cases of several proteins there exist pH stability ranges of the film area, measured at constant pressure, and also pH stability ranges of the film pressure, measured at constant pressure.

These stability ranges are due to association of the molecules in the surface.

The experiments described in this paper were done partly in Prof. Gorter's laboratory at Leyden and partly in Prof. Svedberg's laboratory at Upsala during the years 1934 and 1935.

It is a pleasure to acknowledge my indebtedness for the hospitality afforded in their laboratories.

I also wish to thank the Curators of the Leyden University for a grant from the "Van Vollenhoven Fonds".

REFERENCES

- Calvery (1931). *J. biol. Chem.* **94**, 613.
Palmer (1934). *J. biol. Chem.* **104**, 359.
Pedersen (1936). *Biochem. J.* **30**, 961.
Philippi (1936). On the Nature of Proteins. (Noord-Hollandsche Uitgeversmaatschappij, Amsterdam, Holland.)
Philpot (1935). *Biochem. J.* **29**, 2458.
Scott (1925). *J. biol. Chem.* **65**, 601.
— (1931). *J. biol. Chem.* **92**, 281.
Svedberg & Sjögren (1928). *J. Amer. chem. Soc.* **50**, 3318.
Ter Meulen-Heslinga (1930). *Rec. Trav. chim. Pays-Bas*, **49**, 396.
Wintersteiner & Abramson (1933). *J. biol. Chem.* **99**, 741.