V. THE SPREADING OF COMPLEX PROTEINS.

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SPREADING of proteins can be obtained by bringing a small amount of concentrated aqueous solution of the protein on to the surface of a Langmuir-Adam tray. In our experiments 0.5 % protein solutions and a micro-pipette containing 0.005 ml. are used. The pipette is held horizontally, so that the point is close to the water surface, and the content is slowly blown out. If conditions are favourable for spreading, measurements can be made after a very short time (1-2 minutes).

In order to determine the area occupied by a protein under different pressures, a glass slide is moved towards the floating barrier until this barrier moves away, thus indicating a small rise of the surface pressure.

The floating barrier can now be brought back into equilibrium by torsion of a spring mounted on a balance. The amount of torsion is read on a scale, which is calibrated in dynes per cm. of the floating barrier. The area is measured for different pressures and by extrapolating from these numbers, the area for zero pressure can be found. This part of the technique is identical with that described by Adam [1930] in his studies of the spreading of different substances.

It has been possible to show [Gorter, 1926; Gorter and Grendel, 1926; 1928; 1929; Gorter and Seeder, 1932; Gorter *et al.*, 1932, 1934] that spreading of a protein in a very thin layer (6-7.5 Å. thick) occurs under different conditions. From the experiments of Hughes and Rideal [1932], who used a somewhat different technique, this same thickness can be calculated.

The measured spreading (extrapolated to zero pressure and calculated in sq.m./mg. of spreading substance) is plotted against the p_H of the solution in the tray on which the substance is spread.

The buffers used in the tray were, amongst others, $M/300$ solutions of acetate-acetic acid covering the range p_H 3.6-5.6.

Spreading ovalbumin at a range of these p_H values, a maximum is found at p_H 4.6 (the isoelectric point) and very low minima of spreading on both sides of the isoelectric point, provided the solutions contain very few and only univalent ions.

By increasing the amount of electrolytes in the tray, the minimum spreading on both sides of the isoelectric point can be made to increase.

Multivalent negative ions appear to have a great influence on the minimum on the acid side (p_H 3.0), multivalent positive ions on that on the alkaline side (p_H 6.0) of the isoelectric point. Large amounts of univalent ions (± 40 millimol per litre) small amounts of bivalent ions (± 1 millimol per litre) or very small amounts of trivalent ions ($\pm 1/40$ millimol per litre) all produce similar effects: if they are positive they change the minimum spreading on the alkaline

side into a maximum, if they are negative, they cause the disappearance of the minimum on the acid side.

Not only inorganic ions but also organic ions like glutathione on the acid side, or spermine on the alkaline side, produce the same effect, when added to the solution in the Langmuir tray. Moreover, a difference is seen between the members of a lyotropic series. Thus Li has a smaller influence than K, and more is needed of Cl to produce the same effect than of CNS [Gorter, 1934].

The explanation of these phenomena is that a protein like ovalbumin does not spread well in an ionised state, whereas the undissociated protein and its non-ionised salts do spread. Addition of ions to the water in the tray favours the spreading by discharging the protein ion.

These results have been obtained with different proteins: ovalbumin, pepsin and trypsin have been most completely studied.

It is the purpose of this paper to give the results of investigations of differences in spreading on different solutions in the tray between a simple protein and a complex protein artificially prepared therefrom.

In these complex proteins basic or acidic substances are combined with acid or basic groups of the protein.

The spreading of complex proteins.

Many proteins are complex in so far as they contain a simple protein molecule to which a prosthetic group is attached, which is important for the behaviour of the molecule as a whole.

In order to elucidate the spreading of this group of proteins, we have prepared complex proteins by adding different substances to ovalbumin and to pepsin.

The first protein is very well suited for this study; it contains a great many free amino- and carboxyl groups and therefore the introduction of another molecule attached to these groups will be most likely to influence the mode of spreading at varying p_H .

Pepsin has a pronounced minimum on the alkaline side of the isoelectric point (p_H 2.85) and therefore is very appropriate for studying the influence of positive ions. Ovalbumin can be combined with tartrazin (I).

It is obvious that the latter, being a strong acid, must combine with the free NH_2 groups of the protein.

If we study the spreading of this complex protein, we see no change at the isoelectric point and on the alkaline side of this point, whereas the minimum on the acid side at p_H 2.7–3.0 disappears completely (Fig. 2).

Examination of the amount of tartrazin that must be added to an ovalbumin solution shows that 5.6 mM tartrazin is sufficient for an ovalbumin solution containing 5 mg./ml., this being 1/7 mM (mol. wt. 35,000). From these numbers we may calculate that ovalbumin contains 39 free NH_2 groups per molecule which combine with tartrazin to form a product showing maximum spreading at p_H 3.0.

Fig. 1. The dotted curve shows the spreading of ovalbumin to which no tartrazin has been added. The continuous curve shows the area (in sq. m. per mg.) of the protein when spread on different solutions in the tray, the p_H of which is indicated on the abscissa. This ovalbumin-tartrazin complex contains 5 mg. ovalbumin per ml. and is 16mM with respect to tartrazin, an excess of which is present.

Measurements shown in this and other figures are made after 1-2 minutes unless otherwise stated.

Fig. 2 Showing amount of tartrazin in millimols per litre that must be added to a 0.5 % ovalbumin solution in order to obtain spreading. The areas are indicated on the ordinate in sq. m. per mg.

If a less than 5.6mM solution of tartrazin is added to the same ovalbumin solution, some of the NH₂ groups only are bound and intermediate values for the spreading are obtained (Fig. 2).

The explanation must be that the protein now behaves as an acid and no longer as a zwitterion.

The reverse can be shown when a complex is prepared by adding to pepsin a substance like spermidine (II).

This combines with the free COOH groups and now the curve of the spreading at varying p_H has the form shown in Fig. 3.

Fig. 3. The curve $\circ-\circ-\circ$ gives the area for the spreading of pepsin (Northrop) on solutions of varying p_H . The dotted line between p_H 1.2 and 2.1 is the curve given by pepsin, to which no acetic acid is added as in this experiment (see experimental part).

The curve $\times - \times - \times$ gives the spreading of a pepsin-spermidine complex on solutions of varying p_H .

This is to be explained by the transformation of the protein into a multivalent amine.

Here however on the acid side of the isoelectric point a different spreading is also observed with spermidine-pepsin. This may be due to the fact that pepsin is already a complex protein (see below).

We have also studied the influence of nucleic acid on the spreading of ovalbumin,

and we were unable to discover any definite change of the spreading at varying p_H as is shown in Fig. 4.

This fact is readily explained by the amphoteric character of nucleic acid itself¹. At p_H 3.0 on the acid side of the isoelectric point combination of the

¹ We give the formula of $\frac{1}{4}$ part of nucleic acid (III) to show this.

phosphoric acid groups with the NH_2 groups of the protein is to be expected. But this does not influence the spreading, because the other end of the molecule has a free NH_2 group which simply replaces the original free NH_2 group of the protein. On the alkaline side at p_{H} 6.0, the same reasoning holds good. The free NH_2 group of the nucleic acid then enters into relation with the free COOH

Fig. 4. Nucleic acid in 16*mM* solution is added to ovalbumin. There are only minor differences from the ovalbumin-curve (cf. Fig. 1). Points o and x represent different preparations.

group of the protein, but now the acid character is not modified, the COOH groups being simply replaced by the free acid groups of the phosphoric acid. The same reasoning can explain the absence of different behaviour in spreading experiments with the azoprotein prepared by coupling ovalbumin with the compound

If now we compare the behaviour of this group of complex proteins with that of trypsin, pepsin and insulin, it is possible to state that in the latter proteins only few NH_2 or COOH groups are present.

Pepsin gives the same type of curve as shown by tartrazin-ovalbumin [Gorter and van Ormondt, 1935]; this is in agreement with the small number of free NH_2 groups (5 per molecule of 34,500) found by Northrop [1930] for this protein. Moreover, the curve of acetylpepsin¹ is somewhat modified, the minimum on the acid side of the isoelectric point being less distinct than that of ordinary pepsin (Fig. 5).

The behaviour might be explained by the assumption that pepsin is formed by the combination of a protein with a fairly strong multivalent acid neutralising most of the NH_2 groups of the protein molecule.

The reverse observation can be made with trypsin [Gorter and van Ormondt, 1935] which behaves like a spermidine-protein complex. It is reasonable to

¹ Kindly supplied by Dr J. H. Northrop.

suppose that trypsin is a complex protein consisting of a combination of a protein and a multivalent base.

Insulin [Gorter and van Ormondt, 1933] gives a curve that would indicate that some of the NH_2 groups are not available for reaction with H^+ ions on the acid side of the isoelectric point, because its minimum is not pronounced as in

Fig. 5. The spreading of acetylpepsin. The dotted line gives the spreading of pure pepsin and the continuous line that of acetylpepsin solutions of varying p_{H} .

ovalbumin. It has the form of the curve obtained with a tartrazin-ovalbumin complex formed by admixture of less than $5.6mM$ tartrazin solution with a 0.5% ovalbumin solution.

It is interesting to add to the above-mentioned fact a series of observations showing that not only does ovalbumin combined with tartrazin behave differently from ordinary ovalbumin, but that the same result can be obtained by spreading ordinary ovalbumin at a p_{H} 3.0 on a tray containing tartrazin.

Here the maximum spreading is obtained within a short time after the ovalbumin has been blown out on the surface of the water in the tray containing tartrazin in a $1/80mM$ solution at p_{H} 2.7 (Fig. 6), and the same observation holds good at p_{H} 5.6 when spermidine is added to the water in the tray on which ordinary pepsin is spread, and the spermidine content is mM at p_{H} 5.6 or 7.45 (Fig. 7).

Fig. 8 gives for comparison the amount of spermidine to be added to 0.5% ovalbumin solution in order to obtain a product that shows maximum spreading at p_{H} 5.6.

This effect is no longer observable with the tartrazin-ovalbumin or spermidine-pepsin complex owing to the fact that the NH_2 groups or COOH groups can no longer react.

This influence of substances such as tartrazin on the acid side is exactly comparable with the influence of bivalent negative ions (e.g. SO_4^{2-} or glutathione) whereas spermidine (Figs. 9 and 10) has the same action as bivalent positive ions (e.g. Mg , Sr or agmatine). We can obtain maximum spreading of ovalbumin

Fig. 6. Showing dependence of maximum spreading of ovalbumin on tartrazin at constant p_H upon the concentration of tartrazin.

Fig. 7.

Fig. 8.

Fig. 7. Effect of concentration of spermidine in tray solution upon spreading of pepsin.

Fig. 8. Effect of concentration of spermidine in pepsin solution upon spreading of latter on acetate buffer solution, 0.033 M .

at p_H 3.0 by addition of the negative ions and at p_H 6.0 by adding the positive ions.

Fig. 9. Spreading of ovalbumin on solutions of glutathione of varying concentration. Left-hand curve after 6 minutes; right-hand curve after 12 minutes.

Fig. 10. Continuous curve shows spreading of ovalbumin on glutathione solutions of varying p_H ; dotted curve shows the spreading on water at similar p_H .

The above experiments may indicate that at the water surface compounds of the protein and these different substances are formed.

Preparation of ovalbumin.

The ovalbumin used in these experiments was prepared in the following way.

The white of 20 fresh eggs, usually about 600 ml., was beaten and mixed with an equal volume of saturated ammonium sulphate to precipitate ovoglobulin. After standing 24 hours in the ice-box, the ovoglobulin was removed by centrifuging and to the remaining solution was added, slowly with careful stirring, saturated ammonium sulphate until the liquid became distinctly turbid. The slight precipitate was dissolved by slowly adding just sufficient distilled water. To the clear solution was then added, very slowly with careful stirring, 10 % acetic acid until again a slight precipitate was formed. Then some more saturated ammonium sulphate was added to induce rapid crystallisation. After standing 1 or 2 days in the ice-box a large amount of ovalbumin had crystallised. It was centrifuged and dissolved in distilled water. The solution was again treated with ammonium sulphate and acetic acid in the manner described above, and in all six recrystallisations were usually carried out in about a fortnight.

The solution was then dialysed in the ice-box against distilled water, using a cellophane membrane, until no more sulphate could be detected in the outer liquid. The concentration of the pure solution was determined either by ter Meulen's micro-nitrogen method or Bang's gravimetric method.

Preparation of the azoprotein.

0.46 g. of benzidine was dissolved in 100 ml. of water containing 3 ml. of 1:1 hydrochloric acid and tetrazotised at 7–8° with an aqueous solution of 0.35 g. of sodium nitrite. The solution was poured into a chilled solution of 3 g. of sodium acetate in 500 ml. of water, and to this was added a solution of 0.36 g. of salicylic acid in 100 ml. of water. The mixture slowly became a chocolate-brown colour, especially after adding 20 ml. of $N K_2CO_3$. The intensely coloured solution now contained tetrazotised benzidine coupled on one side with salicylic acid, but with the other diazo-group free.

165 ml. of dialysed egg-albumin free from ammonium salts containing about 1 g. of protein were made alkaline with 15 ml. of $N K_2CO_3$. 130 ml. of the above diazo-solution were then run in followed by 8 ml. of $N K_2CO_3$.

The dye protein solution was next chilled overnight and acidified first with acetic acid and then with hydrochloric acid until acid to Congo red.

The crude salicylic acid-azo-benzidine-azo-egg-albumin was collected by centrifuging. The centrifugate was washed with acidified water and again centrifuged. This was repeated three times. The precipitate was dissolved in distilled water by adding a few drops of 2.5 N NaOH. The p_H of the solution was 11.6; by addition of HCl it was reduced to 8.4.

Preparation of the tartrazin-ovalbumin complex.

Since tartrazin is an acid dye, according to the rule that acid dyes colour best in acid medium [Hammersten *et al.*, 1928], the tartrazin solution and the ovalbumin solution were mixed at p_H 4.0. 4 ml. of egg-albumin solution, containing 60 mg. per ml., were diluted with citrate buffer (McIlvaine) to 44 ml., and to this solution 20 ml. of 1 % tartrazin in 50 % alcohol were added. A yellow precipitate separated and was centrifuged. The precipitate was washed three times with citrate buffer solution (p_H 3.0). The precipitate could be dissolved in a buffer solution of p_H 11.0 (made from 0.1 N NaOH and $M/10$ borax).

The percentage of tartrazin in the solution was determined colorimetrically and the amount of egg-albumin by the micro-Kjeldahl method. The nitrogen content of the solution was determined and from this the nitrogen value of the tartrazin was subtracted.

Ovalbumin with varying amounts of tartrazin, obtained by simply adding different amounts of tartrazin solution to an ovalbumin solution, were made up. The following were prepared: 10 ml. containing 6.2 mg. ovalbumin per ml. and 0.016, 0.008, 0.004, 0.002 and 0.001 M tartrazin respectively. The p_H was about 5.7.

Ovalbumin with varying amounts of nucleic acid.

In a similar way solutions containing ovalbumin and adenylic acid or rather sodium nucleate were prepared.

The following were prepared: a solution containing 6.2 mg. ovalbumin per ml., which was 0.016 *M* with respect to adenylic acid; the other solutions contained the same amounts of ovalbumin but were 0.008, 0.004, 0.002 and 0.001 *M* with respect to adenylic acid.

Pepsin with spermidine.

A solution of pepsin [Northrop, 1930] in glycerol containing 30 mg. of crystalline pepsin per ml. was diluted five times, and 1 ml. quantities of this solution were mixed with varying amounts of 0.032 *M* spermidine and the equivalent amounts of acetic acid. Each mixture was made up to 2 ml. The following were the final concentrations of spermidine: 0.001, 0.002, 0.004, 0.008 and 0.016 *M*.

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