THE SURFACE ACTIVITY OF PROTEINS

HANS NEURATH

Department of Biochemistry, Duke University Medical School, Durham, North Carolina

AND

HENRY B. BULL

Department of Chemistry, Northwestern University Medical School, Chicago, Illinois

Received November 18, 1938

The authors have set themselves the task of reviewing the literature dealing with the surface activity of proteins. In such a review the subject of protein surface films must occupy a central position. To facilitate the presentation of this material, the subject has been divided into two parts. The first part deals with the surface films of proteins formed spontaneously on protein solutions; the second part describes the work done on protein films spread on pure buffer solutions or on pure water. While there is a marked similarity between these two types of films, they are by no means identical.

During recent years there has been a vigorous revival of interest in the subject of surface films in general and protein films in particular. Much of the new work was anticipated, at least in a semi-quantitative way, many years ago, and due credit should be given these early investigators.

I. Surface Films on Protein Solutions

In 1873 Plateau (126) studied, by means of the retardation of the swing of a magnetic needle floating on the surface, the surface viscosity of protein solutions. Even before this time Melsen (106) and Smee (138) showed independently that an insoluble coagulum of protein was formed when protein solutions were shaken. Ramsden (129) in 1894 described experiments in which he was able to coagulate a number of proteins by shaking their solutions. He showed, for example, that egg albumin, egg globulin, vitellin, serum albumin, serum globulin, fibrinogen, lactalbumin, myosinogen, and potato protein all formed insoluble precipitates or coagula when their solutions were shaken. He definitely suggested that the coagula were due to the rolling or heaping up of the protein film at the air–solution surface. He was also able to show that the process was independent of the nature of the gas present.

From the first there seemed to be an appreciation of the importance of

such films to the study of biology. In 1840 Ascherson (6) remarked that oil droplets suspended in albumin solutions had a tough skin or film formed spontaneously around them and suggested such droplets as models for living cells.

In recent years du Noüy (37), followed by several other workers (29, 71), has studied the surface tension of dilute solutions of proteins and attempted to interpret the results in terms of the thickness of the film formed on the protein solution surface. Briefly, these workers claimed to have observed certain very sharp minima in the surface tension of such solutions at certain protein concentrations. They assumed that these minima corresponded to a particular orientation of the protein molecules at the surface. They made the further assumption that all of the protein had been adsorbed at the air–solution surface and glass–solution interface and, further, that the same amount of protein had been adsorbed per unit area on the protein solution–air surface as at the protein solution–glass interface. They then calculated the thickness of the adsorbed film at the various minima in the surface tension–protein concentration curve. From this thickness they calculated the dimensions of the protein molecule. Such measurements and calculations are to be regarded with extreme skepticism. In the first place, it is very doubtful that the minima in the concentration–surface tension curve have a real existence (to be discussed under the section on surface tension), and, secondly, it is very questionable whether the protein is completely adsorbed at the surfaces. Experiments on protein adsorption show that it follows a Langmuir adsorption isotherm (73, 100), and under such circumstances part of the protein would remain in solution. A third difficulty is that with such dilute solutions of protein as were used in these experiments it is almost impossible to know the true protein concentration with any accuracy. A large fraction of the small amount of protein present may have been lost by adsorption on glassware during the preliminary manipulation; also, part of the protein could be lost through surface denaturation. And, lastly, there is no good reason to believe that the minima which were claimed corresponded in any way to orientation on the surface. Certainly the dimensions reported have not been confirmed by the spreading of protein on a buffer solution and measuring the force–area relations of the films.

Wu and Ling (144), following the lead of Ramsden, investigated in a quantitative fashion the coagulation of purified egg albumin, oxyhemoglobin, and methemoglobin brought about by the shaking of solutions of these proteins in glass bottles in a shaking machine. They found, in general, the rate of surface coagulation to be independent of time and concentration but strongly influenced by the rate of shaking and by the size of the bottles. A reaction whose rate is independent of time and

concentration is classed as an apparent zero-order reaction and is characteristic of reactions taking place at a surface saturated with reacting materials. It is to be anticipated that, if the protein concentration becomes too low or the rate of creation and destruction of surface too fast, the reaction would fail to follow a zero order. Bull (19) was able to demonstrate that such was the case. Wu and Ling reported the rate of surface coagulation to be greatly influenced by the hydrogen-ion concentration, the maximum rate for egg albumin being at the isoelectric point and nearly independent of salt concentration. Oxyhemoglobin showed a much more complicated behavior in respect both to hydrogen-ion concentration and to salts. The rate of surface coagulation for this protein showed two maxima,—one at pH 3.9 and the other at pH 6.9. The addition of salt (1 per cent sodium chloride) greatly increased the rate of coagulation and shifted the maximum rates to pH 5.0 and 8.7, respectively. The behavior of methemoglobin, as far as it was investigated, was similar to that of oxyhemoglobin, but the rate of coagulation was somewhat slower. Wu and Ling studied the influence of surface-active materials and showed that alcohol, ether, and saponin greatly diminished the rate of surface coagulation of both methemoglobin and egg albumin. They also reported that the pH values of unbuffered solutions of egg albumin and oxyhemoglobin changed upon shaking. They determined the rate of surface coagulation of egg albumin at 25°C. and 38°C. and reported a Q_{10} over this temperature range of 1.09. Incidentally, they were unable to confirm Hopkins' (74) earlier observation that sulfur is given off as hydrogen sulfide or as some other volatile sulfide when egg albumin solutions are shaken.

Neurath and Bull (119) studied the density of surface-coagulated egg albumin and its water-binding capacity. They found its density to be 1.3016, using xylene in the pyknometer as the displacing liquid. This is a higher density than that of the heat-coagulated egg albumin (1.2940) and considerably greater than that for the native egg albumin (1.2655). They estimated the water binding (hydration) of surface-coagulated egg albumin to be from 0.19 to 0.20 g. of water per gram of protein. Bull (21) determined the density of native, surface-coagulated, and heat-coagulated egg albumin, using hydrogen as a displacing gas, and found that all three forms of this protein had, within experimental error, the same density, which was 1.345 ± 0.005. The different densities reported by Neurath and Bull, using xylene as the displacing liquid, can probably be attributed to lack of penetration of this liquid into the protein.

Bull and Neurath (23), unaware of the work of Wu and Ling described above, investigated the surface denaturation and coagulation of egg albumin produced by shaking solutions of this protein in glass bottles. They

confirmed the results of Wu and Ling on most points, but were unable to agree with the Chinese workers that surface coagulation cannot be separated into two steps in analogy to heat coagulation: namely, (*1*) the production of soluble surface-denatured protein and (*2*) the coagulation of this soluble surface-denatured protein. Bull and Neurath found that away from the isoelectric point, both on the acid and on the alkaline side, a large fraction of the surface-denatured protein was soluble, as evidenced by the fact that, if such solutions are brought to the isoelectric point, a precipitation of the surface-denatured but uncoagulated protein takes place. Wu and Wang (145) challenged Bull and Neurath on this point, but were unable to offer a reason for the disagreement. Bull and Neurath (24) repeated their own experiments and confirmed their previous findings. The reviewers feel that a positive result in this connection is of more significance than a negative one.

Bull and Neurath also investigated the influence of gelatin on the surface coagulation of egg albumin and found that this substance was without effect up to its gelling point. They also studied the change of pH experienced by unbuffered protein solutions upon surface denaturation in some detail and pointed out the close analogy with the behavior of heat-denatured egg albumin solutions in this regard.

Bull (21) determined the rate of surface coagulation of egg albumin solutions at 45°C. and 25°C., and found a Q_{10} over this temperature range of 0.95, which is somewhat less than that reported by Wu and Ling. In any case, however, the temperature coefficient of surface coagulation is very much less than that of heat coagulation and is more in line with that found for urea denaturation of egg albumin (75).

Bull (19) developed an apparatus for the study of protein films on protein solutions which yielded somewhat more quantitative results than it was possible to attain by the bottle-shaking technique. He employed a roller of known dimensions dipping into an egg albumin solution and rotated at known speeds. With this technique it is possible to know accurately the rate and extent of creation and destruction of air–protein solution surface. Then from a knowledge of the amount of surface-coagulated protein in a given time may be calculated the amount of surface-coagulated protein per unit of surface area as a function of speed of rotation and of protein concentration. From a knowledge of the specific volume of the protein, the thickness of the film of denatured protein at the surface is calculated. It was found that the thickness of the film calculated in this manner was a smooth function of the speed of rotation of the drum; this allowed an extrapolation of the thickness of the film to zero speed of rotation to yield an approximate estimate of the thickness of the film on a quiescent solution at the various protein concentrations used. The results of such an

extrapolation indicate a rather complicated picture for the surface film on a quiescent protein solution. As far as can be judged, a layer of denatured egg albumin molecules is immediately on the surface and is about 10 Å. in thickness. Such a film would correspond to a strongly compressed spread film produced by dropping a small amount of dilute protein solution on a pure buffer solution (the properties of such films are to be described in a later section) and involves, as Neurath (115) has suggested, an unfolding of the native molecules at the surface to produce the denatured protein. Under this denatured film is adsorbed a layer of more or less spherical, native molecules which is about 40 Å. thick. The extent of adsorption of this second layer is dependent on concentration, and either does not exist below a concentration of 0.10 per cent protein or else forms relatively slowly so as to be missed by the roller technique, which allows as a maximum only 3 sec. for the formation of the film. An important point brought out in this research is the remarkable rapidity of formation of the surface film. For example, at concentrations greater than 1.0 per cent, the two-layer film is complete in less than 0.7 sec. after creation of the new surface. If the protein concentration is sufficiently great and the rate of creation and destruction of solution surface is not too high, the surface will be completely saturated with protein and the limiting factor in the production of denatured protein is the rate of creation of new surface; accordingly the reaction has the kinetics of that of a reaction of an apparent zero order.

SURFACE TENSION OF PROTEIN SOLUTIONS

It appears that in most cases there is no true equilibrium established between the protein solution and the surface film. Langmuir (98) has emphasized the tremendous insolubility of spread films of proteins and the improbability of any of the protein molecules leaving the surface and reëntering the solution. Such a state of affairs renders the use of the Gibbs' adsorption equation a very doubtful aid in interpreting surface tension data from protein solutions. So far, surface tension studies on protein solutions have not been related except in a very vague fashion to the other physical properties of proteins.

Methods

The surface tension of protein solutions is extremely difficult to measure accurately and, in spite of the very large literature on the subject, it may be fairly stated that the equilibrium surface tension of such solutions has never been determined. It is characteristic of the surface tension of protein solutions to drop progressively with time, and the unstable character of the molecule, together with the marked tendency to become infected

with foreign organisms, precludes the possibility of extended measurements, so that equilibrium observations cannot be made. Quite apart, however, from this serious handicap, there are other factors which limit very seriously the techniques which can be used.

As pointed out in the previous section, an insoluble film is formed at a protein solution surface. It is probable that this film is highly organized, with the protein molecules oriented (10). If the film is disturbed during the course of the surface tension measurement, the film organization is disrupted and the tension measured bears little relation to that in its quiescent condition. This fact alone renders the du Noüy tensiometer more or less valueless for such measurement. Johlin (82) has pointed out how extremely critical the ring and wire diameters are for this method. The use of the ring method (du Noüy) for the measurement of the surface tension of protein solutions is to be discouraged.

The formation of threads of coagulated protein induces inaccuracies in the capillary rise method. The reviewers have observed that even in the absence of protein threads the column of protein solution is apt to stick at points removed from equilibrium in much the same manner that a column of dirty mercury fails to show the true pressure in a mercury manometer. It is evident that, since the surface tension of protein solution changes so drastically with time, such methods as the drop weight and maximum bubble pressure method of Sugden give only a measure of the dynamic tension, which has little significance for the structure of protein films.

The sessil bubble method as employed by Johlin (85) seems to be free from these objections. Unfortunately, this method is relatively insensitive, and Johlin reports also that the values one obtains are, for unknown reasons, apparently too high. The reviewers feel that the method of Wilhelmy (141), as developed by Harkins (69), holds very attractive possibilities for the measurement of the surface tension of protein solutions. Bull (22) has applied this method to determine the surface tension of egg albumin solutions with very gratifying results.

Table 1 shows the attempts which have been made to measure the surface tension of protein solutions. In spite of the relative character of most of these measurements they are, with one or two exceptions, in broad qualitative agreement. We shall now discuss these results with respect to protein concentration, time, pH, and salt concentration.

Protein concentration

In general, as the protein concentration is increased the surface tension decreases, sharply at low concentrations and reaching more or less of a limiting value at higher concentrations. Various workers (29, 37, 71) have reported maxima and minima in the surface tension as protein con-

TABLE 1

Surface tension studies on proteins

PROTEIN	METHOD	VARIABLES	WORKERS
Egg albumin and hemoglobin	Sugden bubble pressure	pH, heat denaturation	Loughlin (103)
Gelatin	Sessil bubble	Time, pH, protein concentration	Johlin (85)
Serum proteins	Capillary rise	CO_2 content	Johlin (83)
Gelatin	Drop weight and capillary rise	pH, time	Johlin (80)
Casein, egg albumin, hemoglobin	Capillary rise	Time, protein concentration	Johlin (81)
Gelatin	Sugden bubble pressure	pH	St. Johnston and Peard (87)
Caseinogen, egg albumin	Sugden bubble pressure	Electrolytes, pH	St. Johnston (86)
Egg albumin, serum albumin, gelatin	Stalagmometer	pH, anions	Errera and Millan (38)
Gelatin	Drop weight	pH, protein concentration	Davis, Salisbury, and Harvey (28)
Gelatin	Stalagmometer	pH, electrolytes	Artom (5)
Gelatin	Capillary rise	pH	Jermolenko (79)
Serum albumin, gelatin, egg albumin	Ring (du Noüy)	Time, pH, protein concentration	de Caro and Laporta (30)
Casein, egg albumin, serum albumin	Stalagmometer	pH, heat denaturation	Berczeller (12)
Egg albumin, serum proteins	Ring (du Noüy)	Time, protein concentration	du Noüy (37)
Egg albumin	Drop weight	pH, time, concentration	Fu and Wu (42)
Various proteins	Capillary rise and stalagmometer	pH	Bottazzi*
Egg albumin	Wilhelmy balance	pH, protein concentration	Bull (22)

* Atti Accad. Lincei **21**, **II**, 221, 561; **22**, **II**, 141.

centration is progressively increased. It is the reviewers' opinion that such irregularities are in most cases artifacts. Certainly the Wilhelmy and sessil bubble methods disclose no such complicated relations. It appears that such irregularities could arise from (*1*) changes in the hydrogen-ion concentration in unbuffered solutions, (*2*) surface contamination by surface-active materials, and (*3*) inherent defects in the techniques employed. It is possible to realize a broad minimum in the surface tension of colloidal substances, such as sodium oleate (83), and presumably such a situation could arise in protein solutions, but the reviewers feel that such a relation has not as yet been established for protein systems. Incidentally, the minimum in the sodium oleate–surface tension curve is attributed to association of particles at higher concentrations and has nothing to do with a change of orientation at the surface. The above remarks apply specifically to the efforts of du Noüy and other workers to evaluate molecular dimensions from surface tension data.

If no molecules were able to leave the surface after having entered, the surface should in time saturate itself at the concentration where there was just a sufficient number of molecules in solution to occupy completely the available surface. Further increase in concentration should produce no further change in surface tension. As far as can be judged from the extrapolation of the surface tension to infinite time (see page 399), this is not the case, since even at relatively high egg albumin concentration (0.2 per cent) the surface tension is still slightly dependent on concentration. Two possible explanations for this dependence suggest themselves: (*1*) when the solution surface is covered or nearly covered with denatured protein film, it is impossible for a molecule entering the film from the solution to surface denature, since it has to expand or unfold to do so, and, accordingly, it remains in the soluble native state, in which condition it can pass again into the solution and thus establish an equilibrium for this part of the film, or (*2*) the soluble native molecules are adsorbed on the bottom surface of the denatured film and affect the surface tension indirectly through their influence on the denatured molecules in the film. It may turn out, however, that if and when true equilibrium surface tension measurements are made on solutions of such proteins as egg albumin, the surface tension of the more concentrated solutions will be found to be independent of concentration.

Johlin (85) found that when equilibrium surface tension values of gelatin solutions are plotted against the logarithm of the concentration, a gentle curve was obtained, which departs from a straight line in both the dilute and the concentrated solutions (equilibrium values were obtained with the sessil bubble method after a 24-hr. wait).

Time

All workers agree that the surface tension of protein solutions changes greatly with time, and that this change takes place over a number of hours. du Noüy (37) reports that surface tension follows the relation

$$\sigma = \sigma_0 e^{-Kt^{\frac{1}{2}}}$$

where σ is the surface tension after time t, σ_0 is the initial surface tension, and K is a constant. Johlin (81) criticized this expression and suggested that the surface tension of protein solutions may, except for a very short interval at the beginning, be expressed as a function of time in the form

$$\sigma = \frac{a}{t^n}$$

where a and n are constants. When log σ was plotted against log t, a straight line was obtained which allowed the evaluation of the constants. Bull, using the Wilhelmy method and solutions of egg albumin in $N/150$ sodium acetate buffer, found that plot of log σ against log t did indeed yield straight lines, but only after about 100 min. If the reciprocal of time be plotted against surface tension, a curve is obtained with a very gentle slope at longer times; such a curve can be extrapolated to zero reciprocal time with reasonable safety, and yields an estimate of the surface tension at infinite time. This technique probably gives the closest approximation for an equilibrium value for the surface tension of protein solutions that is obtainable. By this method of estimation Bull (22) found that above a concentration of 0.02 per cent of egg albumin in $N/150$ sodium acetate buffer at pH 4.90, the surface tension was only slightly dependent on protein concentration (a drop of about 1 dyne per centimeter between 0.02 per cent and 0.20 per cent). The surface tension at 0.02 per cent protein was about 47 dynes per centimeter, which corresponds to a film pressure of 25 dynes per centimeter. Interestingly enough, this is the film pressure at which an inflection in the straight line is observed in the pressure–area curve for a film of spread egg albumin on $N/150$ sodium acetate buffer at pH 4.90. Evidently a film on a protein solution can spontaneously attain the film pressure of a highly compressed spread film.

Some workers (30) report a minimum in the time–surface tension curve. Such a minimum could be occasioned by an association of the protein molecules in the surface, i.e., a reaction between molecules. While it is not possible to say definitely, the existence of such minima in the surface tension–time relation of protein solutions seems improbable. It is more probable that such minima as have been reported are due to film plasticity

incident with the gelling of the film and have nothing to do with true surface tension. Such minima are not found with the capillary rise or the Wilhelmy method, but are frequently observed with the ring (du Noüy) method.

The reason for the prolonged change of surface tension of protein solutions with time is obscure, although two possibilities suggest themselves. It is conceivable that the polypeptide chains are highly organized (10) in the completed film, and it might require considerable time for these unfolded, extended chains to orient themselves in the very viscous surface film into positions which yield the minimum free energy. The second possibility, which may be involved as well as the one given above, is that the surface film is being penetrated by native molecules from the solution, which denature in so far as space is available for them to do so (see page 398). It is certain, at least in the case of egg albumin in appreciable concentrations, that the surface is practically saturated within a few seconds after formation, and native molecules arriving from the interior of the solution would find only a small fraction of the surface available for penetration. Accordingly, only a small fraction of the molecules which strike the surface would be effective in lowering the surface tension. This would be expected to prolong the attainment of equilibrium.

The slow attainment of the final value for the surface tension is definitely not due to a slow rate of diffusion of the protein to the surface, as can be seen from the following approximate calculations.

If all the molecules diffusing to the surface remain at the surface, i.e., if the rate of diffusion is the limiting factor in the attainment of equilibrium, the number of molecules in 1 sq. cm. of the surface after time t is given by the following relation (93)

$$n = 2n_0 \left(\frac{Dt}{\pi}\right)^{1/2}$$

where n_0 is the number of molecules per cubic centimeter and D is the diffusion coefficient (this equation assumes an infinite depth of solution). From spread film studies on egg albumin, we know the number of molecules per square centimeter corresponding to a given film pressure. If we assume, as a first approximation, that the film formed on a protein solution is analogous in this respect to a spread film, we may calculate the number of molecules per square centimeter of solution surface at any time, t, by comparing the surface tension with the film pressure of spread films. Table 2 shows the number of egg albumin molecules arriving at a surface of a 0.05 per cent solution as calculated from the diffusion equation given above. These values are compared with the number per square centimeter of solution surface estimated from the surface tension of 0.05 per

cent egg albumin in $N/150$ sodium acetate buffer at pH 4.90, and the film pressure of a spread film of egg albumin on a $N/150$ sodium acetate buffer at pH 4.90. From these approximate calculations it can be seen that a sufficient number of protein molecules had diffused to the surface in 0.58 sec. to saturate the surface completely; hence it may be concluded that diffusion is not a limiting factor.

Hydrogen-ion concentration

Proteins being ampholytes, it is to be expected that hydrogen ions would have pronounced influence on the surface tension of protein solutions. This, in general, is found to be the case, although the various workers are by no means in quantitative or even qualitative agreement concerning this influence.

TABLE 2

Number of molecules of egg albumin diffusing and on the surface

t IN SECONDS	$n \times 10^{-12}$ DIFFUSING	$n \times 10^{-12}$ ON SURFACE
0.10	2.32	
0.58	2.50	
1.00	7.37	
5.00	16.5	
30.00	40.4	
60.00	57.0	1.85
300.00	127.0	1.96
600.00	180.0	2.04
1800.00	312.0	2.17
3600.00	441.0	2.32
7200.00	625.0	2.38
		2.50

Almost every publication dealing with the surface tension of protein solutions has a section on the influence of hydrogen ions. It hardly seems worth while to review this literature in detail. After considering the matter, the reviewers have decided to limit the discussion to the careful work of Johlin (85), who employed the sessil bubble method to measure the surface tension of gelatin and to the work of Bull (22), who used the Wilhelmy balance method to measure the surface tension of egg albumin solution.

Johlin (85) followed the surface tension of gelatin solutions for 24 hr. at pH values from 0.0 to 12.0. He found the surface tension to drop progressively, although he states that at the end of 24 hr. the system had reached equilibrium, as evidenced by no further fall in surface tension. The pH–surface tension curves at the end of 1 and 3 hr. had the same

general contour, although at the end of 3 hr. the surface tension over the whole pH range was about 5 dynes per centimeter lower than at the end of 1 hr. These curves exhibited a sharp maximum at pH 2.0 with a broad minimum at pH 4.6 (isoelectric zone), a fairly sharp rise to pH 8.0, and a flattening off of the curve beyond this pH. The 24-hr. curve (equilibrium) had an entirely different contour from those of the 1- and 3-hr. curves. There was no maximum in the acid region, the surface tension–pH curve rising from about 45 dynes per centimeter at pH 0 to about 50 dynes per centimeter at pH 3.5, with a flatter portion between this pH and pH 5.0 (isoelectric zone), with a sharp rise at pH 6, and with a broad maximum at pH 9.0. Johlin states that the pronounced fall in surface tension in the acid region may have been due to acid hydrolysis of the gelatin, although it is difficult for the reviewers to see why acid hydrolysis would be expected to lower the surface tension, as amino acids have no pronounced capillary activity. It is possible that the acid may have brought about some structural changes in the gelatin molecules, and it might prove worthwhile to investigate other physical properties of gelatin which had been exposed to an acid medium for 24 hr.

Bull (22), using the Wilhelmy balance method and solutions of egg albumin, followed the surface tension from 1 min. to 3 hr., and, by extrapolating the surface tension–reciprocal of time curve to zero reciprocal of time, estimated the surface tension at infinite time. He studied the region between pH 2.8 and pH 9.5. There was no pronounced change in the contour of the surface tension–pH curves with the passage of time, although the surface tension of the more alkaline solutions tended to drop somewhat faster than in the isoelectric or acid ranges. A sharp maximum in surface tension was found at pH 3.2 with a broad minimum at pH 5.0 (isoelectric point of surface-denatured protein) and with an increase starting at pH 5.5 and continuing to a maximum at pH 8.0, but for the longer time this maximum was lost and the curve flattened off at pH 6.0. The surface tension in the alkaline regions was considerably less than in the acid region. The fact that a minimum was observed in the isoelectric zone indicates that the electrocapillary effects are not important, since from these considerations a maximum surface tension would have been expected at the isoelectric point.

Electrolytes

The reviewers have been unable to find any clear-cut results dealing with the effect of electrolytes on the surface tension of protein solutions, and data are badly needed on this point. One would expect, in general, that the presence of electrolytes would tend to reduce the influence of hydrogen ions and to bring the surface tension of both acid and basic solutions to that at the isoelectric point.

ADSORPTION OF PROTEINS AT A SOLID–SOLUTION INTERFACE

Landsteiner and Uhliz (89) were among the first to study protein adsorption at a solid–solution interface. They worked with the proteins of horse serum and were able to show that various inorganic powders adsorbed or removed a high percentage of the protein from solution.

Michaelis and Rona (108) investigated the adsorption of "peptone" by kaolin and charcoal. They stated that the amount of "peptone" adsorbed is independent of the volume of solution employed. They also noted the irreversibility of "peptone" adsorption and concluded from their experiments that the adsorption of "peptone" is not entirely a physical process but involves a chemical reaction.

Biltz (14) studied the adsorption of "albumin" by cellulose, ferric oxide, and kaolin. He found that the amount adsorbed varied with protein concentration and that the adsorption was nearly irreversible.

During all of these early years there were numerous papers dealing with the stabilizing and sensitizing of colloidal suspensions with proteins. The reviewers cannot deal with this phase of protein adsorption.

Loeb (102) studied the rate of flow of water through collodion membranes that had been allowed to come in contact with proteins. He found evidence for adsorption of proteins by the membranes. Hitchcock (73) extended the measurements of Loeb and measured as well the actual amount of egg albumin and gelatin adsorbed per gram of collodion membrane. The adsorption reaction for both proteins followed Langmuir's adsorption equation rather than that of Freundlich. This might be taken as some evidence of only one layer of molecules in the adsorbed film. Hitchcock also found that the maximum adsorption for both proteins came close to their respective isoelectric points, although the maximum adsorption of egg albumin was somewhat on the basic side of the isoelectric point and was at pH 5.0. This last point may be of some significance, since it is now known that the isoelectric point of surface-denatured egg albumin is at pH 5.02 (112).

Palmer (122) further investigated the adsorption of gelatin on collodion disks and found that, although the amount of protein adsorbed was strongly dependent on the pH at low protein concentrations, above 10 per cent protein the amount adsorbed was independent of the pH. He further was able to show that the presence of salts in protein solutions of less than 10 per cent tended to nullify the effect of pH and to increase the amount of adsorption. He considered that his findings confirmed Loeb's theory of the association of gelatin particles.

Dow (35) extended the protein adsorption studies on collodion disks by measuring the adsorption of egg albumin from 0.10 to 23.00 per cent protein concentration as a function of pH and of salt concentration. He found that at higher protein concentrations (about 8 per cent) the adsorp-

tion curve experienced a discontinuity toward increased adsorption. He observed again a maximum adsorption at pH 5.0 in the absence of salts, while in the presence of sodium chloride the amount adsorbed on the acid side of pH 5.0 became equal to that adsorbed at pH 5.0, and on the basic side of pH 5.0 the amount adsorbed was considerably greater than that at pH 5.0 (about 400 per cent greater). Dow was unable to explain the effect of salts on egg albumin adsorption. Loeb's theory of association of protein particles had been evoked to explain the action of electrolytes on the adsorption of gelatin, and the similar behavior of egg albumin in the presence of electrolytes was somewhat embarrassing, since egg albumin molecules are considered not to be associated. The reviewers are inclined to feel that the action of electrolytes on the adsorption is essentially electrostatic in nature and that electrolytes decrease the electrostatic repulsion between protein molecules, thus allowing them to approach closer on the collodion surface; accordingly, a greater adsorption is experienced per unit area. The electrolytes would also decrease the force of repulsion on the basic side of the isoelectric point between the mutually negatively charged protein and collodion and allow the more capillary-active, negatively charged protein molecules (see page 403) to be adsorbed to the greatest extent.

Dow made the rather extraordinary observation that the adsorption of egg albumin at 25°C. was approximately twice that at 1°C.; it is well known that adsorption reactions in general have a temperature coefficient less than 1.

Incidentally, there is some question in the minds of the reviewers in regard to Dow's work, since, in order to have well-mixed systems, the containers were rocked at the rate of 60 oscillations per minute for 18 hr. Under these conditions it is quite possible that an appreciable quantity of the egg albumin had been surface-denatured, and this would obscure the significance of the experiments.

Langmuir and Schaefer (94) have used an optical method for the determination of the thickness of what they designate as S-layers of protein. These films are produced by placing a drop of protein solution on a wet plate upon which have been deposited A-layers of stearic acid conditioned with aluminum chloride solution. The protein is washed off and the plate dried. Then from the change in color of light given by interference from the top and bottom of the film, the thickness is calculated. They found that such a film formed from a 1 per cent egg albumin solution was 50 Å. in thickness. Tobacco virus protein gave a thickness of 300 Å. on a stearic acid surface conditioned as described above, while on a surface conditioned with egg albumin, the thickness of the S-layer of tobacco virus was 125 Å.

In a later publication Langmuir and Schaefer (95) report that better adsorption is obtained if a barium stearate surface is conditioned with thorium nitrate instead of aluminum chloride. With a 1 per cent egg albumin solution the thickness of the S-layer with thorium nitrate was 48 Å., with aluminum chloride 33 Å., and with thorium silicate 35 Å. They make no comment on the revision of their previous value for the thickness on an aluminum chloride conditioned surface from 50 Å. to 33 Å.

Langmuir and Schaefer (96) have extended their study of S-layers to urease and pepsin. They conditioned barium stearate monolayers with thorium dioxide and stated that if the conditioned barium stearate surface was dried before the drop of urease solution was placed on it, an A-layer of urease was formed instead of an S-layer. The S-layer of urease was 35 Å. thick and had great urease activity. They also formed S-layers of urease by placing a drop of urease solution on a wet, clean glass slide. The slide was washed and dried and heated for 16 hr. at 100°C. The urease film on such a slide retained a strong urease activity.

S-layers of pepsin on conditioned barium stearate were 13 Å. thick (Lilly pepsin) and 29 Å. thick (Northrop pepsin) and retained their milk-clotting properties.

The reviewers find it difficult to evaluate the above experiments. The variable thicknesses encountered with the same, as well as with different, conditioning agents speak for a very ill defined layer of adsorbed protein. The thickness is, in general, not sufficient for a monolayer of native protein, and it is too thick for a monolayer of surface-denatured protein. It is possible that the S-layer is a mixture of native and denatured protein.

Of considerable interest for the subject of protein adsorption is the work of Lindau and Rhodius (100). These investigators used gelatin to determine the adsorbing surface of quartz powder. They accomplished this by adsorbing gelatin on quartz plates of known dimensions, and then, knowing the area which corresponded to a given weight of adsorbed gelatin, they were able to calculate, from the amount of gelatin adsorbed by the powder, the area of adsorbing surface per unit weight of powder. It was necessary in this calculation to assume that the adsorption of gelatin per unit area was the same on the quartz plates as on the quartz powder. Having determined the area of the quartz powder in the above fashion, they were then ready to study the adsorption of egg albumin on the quartz powder.

They found that the adsorption of egg albumin followed Langmuir's adsorption isotherm. They extrapolated the adsorption curve to saturation and were thus able to estimate the amount of egg albumin adsorbed at saturation of the quartz surface. They checked this amount by placing the powder in a concentrated solution (about 1 per cent) of the protein,

washing the powder with distilled water, and determining the amount of egg albumin remaining on the powder. The two methods gave very satisfactory checks. They concluded from these experiments that the protein was irreversibly adsorbed on the quartz. They did not establish, however, that the irreversibly adsorbed protein was the only protein adsorbed, i.e., there may have been a reversibly adsorbed layer on top of the irreversibly adsorbed layer. Using 34,500 as the molecular weight of egg albumin and assuming the closest packing of spheres, they arrived at 43 Å. as the thickness of irreversibly adsorbed protein. This value, they pointed out, agrees well with that reported by Svedberg from ultracentrifugal studies. If one recalculates their data, using the specific volume of egg albumin and assuming complete coverage, a thickness of 24.6 Å. is found for the adsorbed protein layer.

The reviewers consider it rather remarkable that protein adsorption which is apparently irreversible can be described in terms of the Langmuir adsorption equation. As is known, this equation was derived by postulating an equilibrium between particles leaving and entering the surface, and if the particles are irreversibly adsorbed, it is hard to see how such an equilibrium could be established. It may be that it is only when the surface is completely or nearly completely covered with protein that the adsorption becomes irreversible, and that this irreversibility is due to the attraction of the protein molecules for each other as well as for the quartz. In other words, the protein becomes coagulated on the surface. At any point less than saturation, the protein molecules would be separated on the surface and would not coagulate, and, accordingly, could leave as well as enter the surface. The fact, however, that Langmuir's adsorption equation is followed, as is shown not only by the work of Lindau and Rhodius but also by that of Hitchcock *et al.*, constitutes another objection to du Noüy's technique for the estimation of the thickness of the adsorbed layer of protein, since it was necessary for du Noüy to assume that all of the protein had been adsorbed out of solution onto the surface.

Lindau and Rhodius (100) also investigated the hydrophilic properties of quartz powder upon which known quantities of egg albumin had been adsorbed. They found that, up to 50 per cent saturation of the surface, the surface had practically the same hydrophilic properties as that of the uncovered quartz, while at 58 per cent coverage the surface became abruptly very hydrophobic and continued to become progressively more so as the amount of egg albumin on the powder was increased. This finding is extremely interesting; it goes hand in hand with the irreversible character of egg albumin adsorption, since molecules which formed a hydrophobic surface would have little tendency to leave the surface and reënter the solution. It also suggests that it is only when the adsorbing

surface is covered to the extent of 58 per cent or more with protein that the protein is irreversibly adsorbed. The hydrophobic character of the film at higher per cent coverage indicates that in adsorption the polar side chains of the protein are directed toward the quartz with the hydrophobic side chains toward the water. It seems probable that such a drastic distortion of the molecule would lead to denaturation.

Lindau and Rhodius (100) determined the amount of egg albumin adsorbed on quartz powder at pH 3.19 and pH 11. They found that the amount of protein adsorbed at these pH values was much less (23 and 7.3 per cent, respectively, of the isoelectric value) than the amount adsorbed at the isoelectric point; on this basis they assumed that the egg albumin molecule swells away from its isoelectric point and occupies a much larger volume and, accordingly, that it takes less protein to saturate the surface. It is to be regretted that Lindau and Rhodius prepared their egg albumin from a commercial stock of dried powder, so that the purity of their preparation is open to suspicion.

There have been a number of investigations of protein adsorption of a somewhat less detailed nature than those given above. Ettisch, Domontowitsch, and Mutzenbecher (39) studied the adsorption of egg albumin, hemoglobin, and serum albumin on collodion membranes. Paic and Deutsch (120) investigated the effect of hydrogen ions on the adsorption of hemoglobin on kaolin and later (121) the influence of electrolytes on the adsorption of hemoglobin on kaolin. Deutsch (31) extended the work on the adsorption of hemoglobin and later (32) investigated the adsorption of serum albumin on kaolin. Spiegel-Adolf (139) reported on the adsorption of serum albumin, pseudoglobulin, and euglobulin by aluminum hydroxide suspensions and by mastic sols.

ELECTROKINETIC STUDIES ON PROTEIN SURFACES

Electrokinetic technique offers a very elegant method for the study of certain aspects of surface reactions of proteins. The reviewers recommend very strongly the careful work and methods of Abramson (1) and Moyer (111) to those interested in making use of this technique for protein study.

The reviewers cannot attempt to describe the methods or theory of electrokinetics or deal with the electrokinetics of proteins in general, but must confine themselves to a review of that work which throws some light on the surface reaction of proteins.

Brossa and Freundlich (18) apparently were the first to coat suspended particles (ferric oxide sol) with protein (beef serum albumin), to study the electrophoretic mobility of such coated particles, and to realize the nature of the measurements they were making. Loeb (101) later used this method to study the electrokinetic behavior of gelatin; in this work

collodion particles were coated with gelatin. Freundlich and Abramson (43) investigated in some detail the electrokinetic behavior of inert particles placed in various concentrations of gelatin, albumin, and hemoglobin. They were able to show that the mobility of the inert particles was lowered by the presence of 10^{-7} g. of protein per liter of solution, that at 10^{-4} g. of gelatin per liter saturation had been reached, and that the addition of further protein was without effect. In both the Loeb and the Freundlich–Abramson measurements the inert particles were large enough to be visible under the microscope, and the electrophoretic mobility was determined by means of the microscopic method. Since this early work a number of investigators (17, 128) have employed this method. Abramson and Moyer have been especially active in exploring the exact relation between the electrokinetic behavior of dissolved protein and the same protein coated on inert particles of microscopic size.

Apparently in some cases the dissolved protein has the same electrokinetic properties as the adsorbed (serum albumin (113), pseudoglobulin (114)), while other proteins show a difference (egg albumin (112), carboxyhemoglobin (36), and oxyhemoglobin (36)). The question of whether or not the protein shows identical electrokinetic properties in the dissolved as well as in the adsorbed state is important, because it gives us some clue as to whether or not the protein is denatured upon adsorption. Thus, egg albumin in the dissolved state is isoelectric at pH 4.52 (112), and the surface-coagulated protein is isoelectric at pH 5.02 (112), while inert particles coated with egg albumin are isoelectric at pH 4.82 (112); this indicates that adsorbed egg albumin may be partially denatured. It may be that the intermediate value is due to the adsorption of some native as well as denatured protein. It would be interesting in this relation to wash the adsorbed surface thoroughly and redetermine the isoelectric point. It might be found that the isoelectric point had risen to that of the surface-coagulated protein.

The work of Dummett and Bowder (36) is hard to interpret, owing to their unfortunate mistake of using the hydrodynamic equations for a flat electrophoretic cell instead of those for a cylindrical cell, with which they worked. The isoelectric points, however, should be reliable, and these indicated that both carboxyhemoglobin and oxyhemoglobin had undergone considerable change upon adsorption (except at a copper surface). Dummett and Bowder reported that adsorption on the inert particles proceeds very quickly and that in 0.01 per cent gelatin equilibrium is reached in a few seconds, while with 0.0012 per cent gelatin equilibrium is reached in about 3 min. They also reported that the nature of the underlying surface is very important in influencing the electrokinetic behavior of proteins, but, owing to their failure to use the proper hydrodynamic

equations, their results in this respect cannot be properly evaluated. Moyer (112) found that the electrophoretic mobility–pH curve for egg albumin is rotated somewhat around the isoelectric point for different surfaces (aluminum oxide, quartz, and silica gel), but that the isoelectric point is independent of the nature of the surface.

It is very curious that the microscopic, inert particle covered with protein behaves electrokinetically as if it had the radius of a protein molecule and not its own radius (114). The reason for this fortunate but surprising behavior is unknown.

Martin (105) studied the rate of adsorption from 0.0002 per cent gliadin solutions on a fritted-glass diaphragm by means of streaming potential measurements. Equilibrium as judged by the streaming potential was reached in from 10 to 50 min. after the glass had come in contact with the protein solution. His data indicate no clear relation between the time for the streaming potential to reach equilibrium and the sign or magnitude of the electrokinetic potential.

Kemp and Rideal (88), using the Mattson cylindrical cell, studied the electrophoretic mobility of quartz powder suspended in gliadin solutions in respect to time, protein concentration, salt concentration, and pH. They found that for a given pH the mobility follows the equation:

$$\Delta u = \frac{KC}{1 + K'C}$$

where K and K' are constants, C is the gliadin concentration in grams per cubic centimeter, and Δu is the difference in mobility between that of the uncoated quartz and of the quartz in equilibrium with a given protein concentration; mobility is expressed in centimeters per second per volt per centimeter. The relation is analogous to a Langmuir adsorption equation and indicates a possible proportionality between mobility and the amount of protein adsorbed. Making such an assumption Kemp and Rideal found that the adsorption of gliadin on quartz was reversible, and that the rate of adsorption followed a unimolecular reaction rate. The adsorption of the positively charged gliadin (pH 3.7) on negatively charged quartz was considerably faster than that of negatively charged gliadin (pH 5.66) on negatively charged quartz. This indicates that, while the electrokinetic potential is important in protein adsorption, it is not the only factor to be considered.

II. Spread Protein Monolayers

Devaux (33) was, apparently, the first to spread protein ("albumin") on pure water and to study the properties of such spread films. He found a film thickness of from 30 to 80 Å.

Metcalf (107), in a brilliant paper in 1905, described the study of "peptone" films spread on water. He devised several types of surface viscometers which he employed to measure the viscosities of spread films of peptone.

During the last decade considerable information has accumulated regarding the properties of spread monolayers of proteins. The fact that before spreading many proteins are soluble in the medium upon which they are to be spread, while the spread film of protein is insoluble, suggests that, in general, proteins undergo drastic structural changes during spreading. Investigations of spread films of proteins have furnished valuable data for the interpretation of protein structure. A notable fact arising from this film work is that intrinsic differences between native proteins largely disappear upon spreading. Thus, the thickness of protein film is approximately the same for all proteins, whereas proteins in the native, dissolved state differ greatly in their molecular dimensions.

The properties of spread protein monolayers and the methods used for their investigation will be discussed in the following sections.

Technique of spreading

Proteins are dissolved in a suitable solvent (usually water) and the protein solution transferred to the surface on which spreading is to occur. As most proteins are water-soluble, there is considerable danger of a portion of the protein being lost in the underlying liquid, and accordingly very exact and careful technique has to be followed in placing the protein solution on the surface.

Gorter and Grendel (51, 54) blow about 0.005 cc. of a protein solution out of a micropipet held horizontally along the surface. Neurath (115) found that Gorter and Grendel's method may be safely modified by allowing larger volumes of a more dilute protein solution to be dropped on the surface from a height of a few millimeters. The method used by Bull (20), at the suggestion of Dr. D. R. Briggs of the University of Minnesota, consists in dipping a calibrated 1-cc. pipet vertically into the surface. After withdrawing it slightly, so that a cone of liquid is formed around the tip of the pipet, the protein solution is forced out gradually by means of a screw arrangement. Apparently, as the film spreads out from the spot where the protein has been applied it drags water along with it and produces a convection upward and along the surface. This convection tends to bring into the surface any material which had passed into the bulk solution.

Hughes and Rideal (77), Hughes (76), and Fosbinder and Lessig (40) found that solid particles, even of water-insoluble protein, spread very rapidly when carefully placed on the surface. The amount of spread

material is determined by means of a modified Nernst balance. As dissolution occurs very much faster at the edge of the solid particle exposed to the surface than from parts of the particle immersed in the water, the risk that protein is lost in the interior of the solution is very small (3, 25).

Philippi (125) has called attention to the discrepancy between the film areas of gliadin as spread by Gorter and Grendel's method, on one hand, and those spread by the method of Hughes and Rideal, on the other. Recent investigation of this problem by Mitchell (109) shows that the area of protein films spread from solution varies with the protein concentration of the spreading solution and attains maximum values only with very dilute solutions. If sufficient time for complete spreading is allowed, monolayers of gliadin spread from a 0.01 per cent solution are almost identical with those obtained by spreading from solid protein.

Homogeneity of protein films

To be able to interpret data obtained from a study of spread monolayers, it is necessary to be certain of the homogeneity of the spread films. The results found by various experimenters working with the same protein sometimes differ widely from one another, and the suspicion arises that the lack of agreement is due to incomplete or imperfect spreading. If the film area is too large, it may be due to contamination of the surface by other surface-active materials or by dust, while too small areas result from incomplete spreading or collapse of the film under unduly high pressures.

Hughes and Rideal (77), Philippi (124), and others have employed surface potential measurements to test film homogeneity. In the case of homogeneous films the surface potential does not vary more than 2 to 3 millivolts as the electrode is moved over the monolayer. Fluctuations in the surface potential from place to place on the film have been found to occur at very low pressures (77), or if the pH and electrolyte concentration of the underlying solutions were not well chosen (59, 124). Also, compression of the film prior to complete spreading may lead to large fluctuations of the surface potential.

Zocher and Stiebel (146) developed a very ingenious method of testing film homogeneity. They observed the film, which was illuminated by light coming through a dark field condenser of the cardioid type located in an opening in the bottom of the trough, with a microscope. It is easy to detect film inhomogeneities with this method, since light is not scattered by a homogeneous monolayer under these conditions, while any unspread material or aggregates formed incidental to collapsing of the film show up as brilliantly illuminated regions. By moving the film under the microscope by means of two rigidly connected barriers, a large portion of the film

can be examined. A simplification of Zocher and Stiebel's method has been introduced by Adam (2). Hughes and Rideal (77) found films of gliadin, glutenin, and egg albumin to exhibit brilliant collapsed patterns. If the collapsing occurred during compression, long folds perpendicular to the direction of compression were in evidence. Similar patterns have been observed by Neurath (115) with serum albumin at pH 4.8 and at film pressures greater than 14 dynes. Zocher and Stiebel reported that solid films of serum globulin were very inhomogeneous, as indicated by optical examination. Casein was found to form homogeneous films when spread on $N/10$ hydrochloric acid, whereas numerous small colloidal particles, imbedded in the solid film structure, could be detected if this protein were spread on distilled water.

Influence of time on the spreading of proteins

The time necessary for complete protein spreading is, in general, longer than that required by other film-forming substances. Gorter and Philippi (59) and Philippi (124) have followed the rate of spreading of egg albumin by means of surface potential measurements. Owing to the fact that the surface potential of a clean water surface differs greatly from the surface potential of the film-covered surface, the film boundary can be readily detected. The time of spreading was found to vary greatly with the pH and electrolyte concentration of the underlying solution. A minimum value of 2 min. was observed at the isoelectric point, while on the acid side the spreading time increased with decreasing pH, and attained a maximum value of 2 hr. at pH 3.80. The spreading time decreased again at pH values below this point, and at pH 1.07 spreading was complete after 10 min. On the alkaline side the spreading time increased with increasing pH, and 9 hr. were required for complete spreading at pH 5.72. If the pH was higher than 6.0, no homogeneous film could be obtained. Philippi's observations strongly suggest that the variation of film areas with pH reported by many authors for a large number of proteins (see following sections) is due to incompletely spread films resulting from insufficient time being allowed for spreading, and that likewise the lack of spreading at the isoelectric point of some proteins (137) may be due to similar causes.[1]

The influence of pH on spreading time can be greatly diminished by the addition of electrolytes. At pH 3.9, for instance, the addition of $N/80$ potassium chloride decreases the spreading time of egg albumin from 2 hr. to 5 min., and at pH 5.7 from 9 hr. to 2 hr. (125). Anions are more effec-

[1] Bence-Jones protein, for instance, continues to spread even 5 hr. after placing the material on an isoelectric buffer solution. Also, urease spreads very slowly under similar conditions (56).

tive in reducing the spreading time of positively charged protein than are cations, and polyvalent anions are more effective than univalent anions. Cations have a greater influence on the alkaline side of the isoelectric point than do anions. Philippi's results indicate that it is the net charge on the protein molecule that determines the rate of spreading; the greater the net charge, the smaller the rate of spreading. The presence of a net charge on the protein molecule tends, apparently, to prevent the molecule from passing from the bulk of the underlying solution into the surface and is, no doubt, due to mutual repulsion of the electrostatic charges of like sign.

Surface pressure measurement of protein films

Before discussing the properties of protein monolayers, it may be well to survey briefly the methods employed for their investigation. Most of the experimenters have used a surface balance of the type described by Langmuir (90), Adam (3), and Harkins (70). A simplified type of Adam's balance has been described by Gorter (62); it lacks, however, the accuracy of Adam's instrument in the lower pressure region. The Central Scientific Company manufactures a surface balance under the trade name of the Cenco hydrophil balance, which yields satisfactory results if not too high accuracy is required.

A very simple method for measuring the surface pressure of films of fatty acids, proteins, etc., has been reported by Blodgett (15). Certain types of oil, such as oxidized lubricating oil, have the property of spreading into thin films which exhibit interference colors. The higher the degree of oxidation of the oil, the thinner the film will be. Such oils are called indicator oils. Very thin films are colorless, while brilliant interference colors appear as the film thickness is increased. Calibration of the interference colors against film pressures, by means of a surface balance, gives a convenient method of measuring film pressures up to 10 dynes. When protein is placed in the center of an indicator oil film, the protein spreads out, displacing the oil and leaving a clear, colorless area occupied by the protein. Upon lateral compression of the combined oil–protein film, successive interference colors appear on the oil film and indicate the exerted film pressure (90). One point about the indicator oil technique troubles the reviewers; is there complete protein spreading under the film pressure exerted by the oil film? Protein films produced under these circumstances may not be entirely homogeneous.

Gorter and Seeder (60) have suggested a method for measuring film areas at zero compression. By means of an electrically operated tuning fork, waves are produced on the surface, and their dampening due to the presence of the film is observed. The interpretation of the results is,

however, so difficult that this method has hardly been used for protein work.

PROPERTIES OF SPREAD PROTEIN FILMS

Spreading power

Comparison of the spreading tendency of various proteins reveals that not all proteins spread with equal ease at their respective isoelectric points (125). Egg albumin (20, 59, 65, 124, 125, 136), serum albumin (53, 115), insulin (63), pepsin (47, 125), zein (63), and others spread readily and completely, whereas proteins such as the respiratory protein of *Palinurus vulgaris* (125), fibrinogen (57), myosin (64), and tobacco virus (137) spread very poorly, if at all, when placed on the surface of an isoelectric buffer solution. Heat-coagulated egg albumin and serum albumin also spread very poorly, if at all (115). If the underlying buffer solution be adjusted to a pH outside of the stability range of the respective protein, a non-spreading protein can usually be induced to spread (50, 125). Philippi (125) has classified proteins into two classes in respect to spreading power: (*1*) those which spread on a buffer at the isoelectric point and (*2*) those which do not spread at the isoelectric point, but do spread outside of their stability region. The reviewers feel that the failure of some proteins to spread is due to the intramolecular cohesive forces being too strong to permit unfolding of the polypeptide chains on the surface.

The addition of a small amount of a proteolytic enzyme induces spreading of the non-spreading proteins. Thus, trypsin enhances the spreading of fibrinogen (57), myosin (64), and denatured egg albumin (49). If the enzymatic action proceeds too far, spreading becomes incomplete (49, 64), owing, no doubt, to the great solubility of the split products resulting from protein hydrolysis.

A lack of spreading has also been observed with gelatin and protamins (50). These proteins have a very high solubility in water, and it appears that dissolution in the underlying solution occurs before unfolding on the surface can take place.

Pressure–area relations of isoelectric protein films

By plotting the pressure exerted on a monolayer against the area of the film, a curve is obtained which can be interpreted in terms of the structure of the monolayer (3). Such curves are so similar for the various proteins that identical film structures for all proteins have been postulated (40, 44, 53, 77). Generally, the compression curves for protein films may be roughly divided into two regions. The first extends from zero compression to about 3 dynes, and the second, which is the high compression region,

from 3 dynes up to the collapse pressure. Proteins such as serum albumin, lactoglobulin (125), and gliadin (77) exhibit, however, another region at very low pressures (0 to 0.3 dyne), where the surface pressure is independent of film area. Such proteins expand indefinitely at zero pressures. Egg albumin and pepsin, on the other hand, do not spread over the entire surface at zero pressure but form rigid coherent films (125). Whether or not such protein membranes form, depends, in the reviewers' minds, merely on the strength of the cohesive forces acting between the polar side chains of the protein film and on the degree of "fitting" of these side chains, rather than on any fundamental difference of structure between these two groups of protein films. The so-called surface patterns of proteins, described by Langmuir (92), seem to be due to similar causes.

At pressures between 0.2 and 1 to 2 dynes the slope of the pressure–area curve increases rapidly. Films which are in the liquid state when completely expanded may become solid when the film pressure attains these values. Measurements of the surface potential, to be described in a following section, reveal that the change of slope of the force–area curve in this low pressure region is due, not merely to packing of the protein molecules, but also to a change in the orientation of the side chains of the amino acid residues. This change of orientation is, apparently, complete when the force–area curve becomes linear with increasing pressure. This linear portion, which is really only approximately linear and is the second region mentioned above, begins at about 3 dynes and extends to about 20 dynes. In this high-pressure region hysteresis (41, 77, 125) of the film has been observed. It is not associated with changes in the surface potential, and equilibrium pressures are usually attained after a few minutes (41).

It has been the custom to extrapolate this linear portion of the force–area curve to zero pressure and characterize the protein film by the area found by such an extrapolation. The reviewers feel that such an extrapolation has no theoretical justification, and a far more significant area would be the area found by extrapolating the pressure–area curve in the lower pressure region to zero pressure or, better still, to consider the area of the film where the coefficient of compressibility is at a minimum. Since, however, most workers have published their results in terms of the extrapolation of the linear part of the pressure–area curve to zero pressure, we shall, for convenience, summarize their findings on this basis. This is done in table 3 for proteins spread on buffers at the isoelectric points of the proteins.

Table 4 shows the corresponding data for proteins which can be spread only outside of their stability zones or by the addition of proteolytic enzymes.

TABLE 3

Limiting areas of isoelectric protein films, obtained by extrapolation of the linear part of the force-area curve to zero pressure

PROTEIN	COMPOSITION OF MEDIUM	pH	AREA PER MILLIGRAM IN SQUARE METERS	OBSERVER
Egg albumin	*N*/300 acetate buffer	4.8	0.88	Gorter, van Ormondt, and Dom (65)
Egg albumin	*N*/20 acetate buffer	4.8	0.88	Neurath (115)
Egg albumin	*N*/300 acetate buffer	4.8	0.93	Ter Horst (140)
Egg albumin	*N*/300 acetate buffer	4.5	1.06	Philippi (125)
Egg albumin	*N*/150 acetate buffer	4.9	1.04	Bull (20)
Egg albumin, heat-denatured	*N*/150 acetate buffer	4.9	0.99	Bull (20)
Egg albumin, urea-denatured	*N*/150 acetate buffer	4.9	0.98	Bull (20)
Egg albumin, urea-denatured	Citrate-phosphate buffer	7.0	1.84	Lee and Wu (99)
Egg albumin	*N*/300 acetate buffer	4.6	1.10	Fourt and Schmitt (41)
Serum albumin	*N*/20 acetate buffer	4.8	1.04	Neurath (115)
Serum albumin	*N*/10 KCl + HCl	4.36	1.08	Philippi (125)
Pepsin	*N*/100 KCl + HCl	2.80	0.92	Philippi (125)
Pepsin	HCl	2.90	1.00	Gorter (47)
Pepsin	HCl	2.70	1.10	Seastone (137)
Trypsin		7.0	1.00	Gorter (47)
Lactoglobulin	*N*/200 acetate buffer	5.3	0.98	Philippi (125)
Insulin	*N*/300 acetate buffer	5.4	1.07	Philippi (125)
Insulin	*N*/300 acetate buffer	5.0	0.88	Gorter and van Ormondt (63)
Oxyhemoglobin	Phosphate buffer	6.8	0.60	Gorter and Grendel (44)
Gliadin	Phosphate buffer	5.9	1.1	Hughes and Rideal (77)
Zein		5.3	1.1	Gorter and van Ormondt (63)
Cytochrome C	K_2CO_3	10.0	1.2	Harkins and Anderson (68)
Insulin*	*N*/100 H_2SO_4		1.75	Mitchell (109)
Insulin*	*N*/100 H_2SO_4		1.23	Mitchell (109)
Gliadin*	*N*/100 H_2SO_4		1.18	Mitchell (109)
Zein*	*N*/100 H_2SO_4		1.50	Mitchell (109)

* Spread on the acid side of the isoelectric point.

Although the film areas given in tables 3 and 4 seem to be nearly the same for all proteins, closer inspection of the pressure-area curves reveals some deviation from such a uniform behavior. As the slope of the pres-

sure–area curve is indicative of the compressibility of the film, it is of interest to compare the compressibilities of a number of proteins with one another. The coefficient of compressibility is defined as

$$-\frac{1}{A_0}\left(\frac{dA}{dF}\right)$$

where A_0 is the film area found by extrapolating the linear portion of the pressure–area curve to zero pressure and dA/dF is the reciprocal of the slope of the linear portion of the pressure–area curve (20). Compressibilities expressed in this manner vary from 16×10^{-3} cm. per dyne for egg albumin to almost twice this value for gliadin. The compressibility

TABLE 4

Limiting areas of protein films. Spreading induced by acids or by enzymes

PROTEIN	SPREADING INDUCED BY	pH	AREA PER MILLIGRAM IN SQUARE METERS	OBSERVER
Myosin	Trypsin	7.3	1.0	Gorter and van Ormondt (64)
Fibrinogen	Trypsin	4.7	0.7	Gorter, Maaskant, and Van Lookeren Campagne (57)
Casein	Hydrochloric acid ($N/10$)		1.0	Gorter and Grendel (52)
Bence-Jones	Hydrochloric acid	1.0	0.8	Gorter and Maaskant (56)
Urease	Hydrochloric acid	1.0	1.0	Gorter and Maaskant (56)
Hemocyanin Limulus	Hydrochloric acid	1.0	1.4	Seastone (137)
Vaccinia	Hydrochloric acid	1.0	0.7	Seastone (137)
Heat-denatured egg albumin	Pepsin		About 1.0	Gorter (49)
Nerve protein	Alcohol	4.6	0.80	Fourt and Schmitt (41)

of protein films probably reflects in some manner the interaction between side chains of the amino acid residues in the protein molecule. The reviewers feel that it would be very profitable to calculate the compressibilities over the entire pressure range. With this mode of calculation A_0 would be the area of the film at the point at which the slope of the line is determined. Making such calculations for egg albumin spread on $N/150$ acetate buffer at pH 4.9, a distinct minimum in the coefficient of compressibility is found at 14 dynes. This point undoubtedly corresponds to fundamental changes in the film. Incidentally, the minimum compressibility is reached considerably before the pressure–area curve begins to depart noticeably from its linear course.

Collapsing of isoelectric protein films

Monolayers of proteins collapse at lower pressures than those of most substances. Indications of collapsing may be furnished by optical examination, surface potential measurements, or by a flattening out of the pressure–area curve at high pressures (68). As the critical pressure which produces collapsing may greatly depend on the rapidity of compression, pH (68, 115), temperature (140), and the presence of impurities (140), the collapsing pressures recorded in the literature are not very significant. The film areas which correspond to the collapse point are presumably the smallest area which the protein molecule can occupy in a homogeneous monolayer, and, accordingly, collapse points are of considerable significance when determined unambiguously (3, 68). It may turn out that the point of minimum compressibility defines with exactness the

TABLE 5

Compressibilities of protein films spread on isoelectric buffer solutions

PROTEIN	COEFFICIENT OF COMPRESSIBILITY IN CENTIMETERS PER DYNE	CALCULATED FROM DATA OF
Egg albumin	0.0160	Philippi (125), Bull (20)
Egg albumin	0.0145	Neurath (115)
Serum albumin	0.0242	Philippi (125)
Serum albumin	0.0230	Neurath (115)
Lactoglobulin	0.0187	Philippi (125)
Gliadin	About 0.028	Hughes and Rideal (77, 3)
Cytochrome C	0.029	Harkins and Anderson (68)
Palmitic acid	0.0021	Adam (3)

collapsing pressure. The collapse pressure of a protein film is greatest at its isoelectric point. Thus, films of egg albumin collapse below pressures of 15 dynes at pH 4.4, and films of serum albumin at 11 to 12 dynes when spread under similar conditions (115). Gliadin collapses at 15.8 dynes, zein at 10.5 dynes, and insulin at 10 dynes when spread on $N/100$ sulfuric acid (109).

Bull (20) has extended surface pressure measurements of egg albumin to pressures as high as 40 dynes. At about 25 dynes the pressure–area curve swings up again. The hydrophilic properties of the film, as measured by the adhesion tension for water of the film deposited on glass slides under various pressures, remain more or less constant from 25 to 31 dynes. Above 31 dynes the film becomes suddenly more hydrophobic. Upon reëxpansion of these highly compressed films and extrapolation to zero pressure, about 30 per cent of the material was found to have been lost, either by dissolution or by aggregation into polymolecular aggregates.

Influence of temperature

Ter Horst (140) has carried out interesting investigations of the influence of temperature on the properties of compressed, and of uncompressed but coherent, films. Observations made 2 hr. after spreading show that increase of temperature produces an increase of film area, irrespective of pH. The temperature had its least influence at pH 2.8 and its greatest at the isoelectric point and at pH 1. It is interesting to note that the influence of temperature is greater on fully spread compressed films than on the uncompressed but coherent film. From the readings it would appear that at about 50°C. the areas of the compressed film (obtained by extrapolation of the linear part of the compression curve to zero pressure) and of the coherent film will be identical. Increase of temperature apparently decreases the angle of tilt of the side chains to the surface, and at sufficiently high temperatures they are lying flat on the surface both in the compressed and in the uncompressed states.

Influence of pH and electrolyte concentration of the underlying solution

Gorter and his associates introduced the term "spreading number" to designate the film area in square meters per milligram of protein. The area is found by extrapolation of the linear part of the pressure–area curve to zero pressure. By measuring the pressure–area relations at a standard short time after placing the material on the surface, a spreading number is obtained which is suitable for comparative purposes. The spreading number was found by these workers to vary with the pH of the trough solution. As a rule, if the spreading number is plotted against pH, a W-shaped curve is obtained. A maximum value is observed at the isoelectric point with minima on both the acid and the alkaline side of the isoelectric point.

Such curves, or at least indications of them, have been observed with egg albumin (61, 65), pepsin (47), trypsin (47), casein (52), zein (63), oxyhemoglobin (44), insulin (63), cytochrome C (68), and others. Also, the tripeptide of α-aminocaprylic acid shows a similar dependency upon pH. The extent of variation of the spreading number with pH varies from protein to protein, and is probably intimately connected with the number and strength of the ionic groups of the protein molecule. Gorter and his associates also found that, at pH regions in which little spreading occurs, maximum spreading may be obtained merely by the addition of salts. It was found that the salts followed the order of the lyotropic series (46) in their ability to increase spreading. The influence of pH can also be greatly diminished, and even completely suppressed, by combining the protein with certain organic acids or bases, prior to spreading.

Thus, the addition of tartrazine (66) restores completely the spreading of egg albumin on the acid side of its isoelectric point, and the addition of spermidine (66) aids the spreading of pepsin on the alkaline side of the isoelectric point of this protein. Protamine is effective in suppressing the pH influence on insulin (45). From the point of view of spreading power, such combined proteins behave no longer as zwitter ions but as polyvalent cations or anions, respectively (49). Mere addition of a proper amount of the organic compounds to the trough solution produces similar effects (66). The increased expansion of monolayers of myosin when spread on potassium lactate solutions has been reported by Moss and Rideal (110).

Gorter and Philippi's observations on the influence of time on the spreading of proteins (see page 412) threw new light on this problem. If sufficient time, prior to compression, is allowed for spreading to become complete, the spreading of a given protein is independent of the pH and the ionic strength of the underlying solution. It is only if compression of a film is started before complete spreading that the spreading number is small. It is true, however, that under certain conditions the spreading process is so slow that complete spreading cannot be obtained.

There exists, however, a considerable influence of the pH and the ionic strength of the underlying solution on the area of completely spread, uncompressed, or slightly compressed protein films. It has been mentioned previously that certain proteins, such as egg albumin and pepsin (125), form coherent, monomolecular membranes of considerable rigidity, even at very low pressures. The area of these coherent films has been found to be constant over a pH range of 4 to 5.5 for egg albumin. At pH values lower than 3.9 the area increases with decreasing pH, the films becoming less homogeneous and less rigid (124). On the alkaline side of this "stability zone" the film area decreases with increasing pH. Proteins which do not form rigid coherent films at low pressures show a similar pH dependency if they are compressed to an area which is greater than that found by extrapolation of the linear part of the compression curve to zero pressure. In these cases, however, the area increases on either side of the isoelectric point. Serum albumin exhibits a "stability zone" between pH 4.9 and pH 8, whereas in the case of insulin the pH range of constant film area is very narrow, around pH 5.5. The pH dependency can also be observed by keeping the film area constant and recording the film pressures in relation to pH (125).

The increase of film areas with pH may be related to the ionization of the polar groups of the film molecules. It will be remembered that ionization of the end groups of long chain acids or amines increases tremendously the expansion of monolayers of these substances. The formation of gaseous films, under these conditions, has been correlated with the repulsive

forces acting between the ionized groups of the molecules (3). Although in the case of protein films the end groups are not free to move about on the surface independently, there is ample evidence to believe that they lie flat on the surface at low pressures. The variations of film areas with pH may, therefore, be ascribed to repulsive forces acting between ionized end groups of these side chains. It is only if the net charge of these zwitter-ionic groups is sufficiently high that the effects of the pH on the film areas become noticeable.

Surface potential measurements

Measurements of the change of the potential difference between a liquid and air, produced by the presence of a monolayer, have been used frequently for investigations of surface films. The arrangement generally used consists of a movable, radioactive electrode above the surface, a reversible electrode in the trough solution, and a potentiometer–electrometer circuit which permits measurements of the surface potential difference between the air–liquid interface (3). Detailed descriptions of the apparatus have been given by Schulman and Rideal (134), Adam and Harding (4), Harkins (67), Philippi (125), and others. The surface potential difference between a clean surface and a film-covered surface may be expressed by the equation:

$$\Delta V = 4\pi\mu n \tag{1}$$

where ΔV is the potential difference in millivolts, n the number of molecules per unit surface, and μ the vertical component of the dipole moment of the film molecules. This equation is an approximation, as it neglects the forces between film molecules and water molecules, the interaction between neighboring film molecules, and the rearrangement of dipoles and ions of the underlying solution (3). In spite of these limitations, determinations of μ from surface potential measurements have furnished suggestive data concerning the rearrangement of film molecules during compression. If no rearrangement takes place, μ should be constant within a certain compression range. Variations may be ascribed to a change of the angle of tilt of the film molecules to the surface, according to the following equation:

$$\mu = \frac{\bar{\mu} \cos \theta}{D} \tag{2}$$

where μ is the observed dipole moment, $\bar{\mu}$ the actual dipole moment, θ the angle of tilt, and D the dielectric constant of the medium. The influence of rearrangement of water molecules and ions and of the polarization of

oriented molecules by neighboring molecules is hardly evaluable and presents a serious difficulty in the interpretation of surface potential data.

Rideal and his associates (76, 77, 135) and Gorter and his school (59, 124, 125) have applied surface potential measurements to protein monolayers. On the basis of equation 1, the surface potential plotted against film areas should give a straight line as long as no rearrangement of film molecules occurs. Adam (3) has recalculated Hughes and Rideal's (77) data on gliadin spread on $N/100$ hydrochloric acid and found μ to be constant at areas beyond 1.3 sq. m. per milligram. Likewise Philippi (125) found values of $\Delta V \times A$ to be constant if the area of monolayers of serum albumin or lactoglobulin was greater than 1.0 sq. m. per milligram of protein. Similar results have also been reported for egg albumin (124). At pressures above about 1 to 2 dynes per centimeter the slope of the potential–area curves decreases with increasing pressure. This change of the slope has been ascribed to a change of orientation of film molecules, as well as to dehydration occurring during compression. Philippi (125) believes that the change of μ upon compression is mainly due to dehydration of the film. In an earlier publication Philippi (124) supported the view of Hughes and Rideal (77), which has been shared by Adam (3), Ter Horst (140), Mitchell (109), and others, that the change of the dipole moment is mainly due to side chains being forced out of the plane of the surface during compression. This view is also shared by the reviewers and will be discussed in a following section (see page 426).

The surface potential of protein films varies markedly with the pH of the underlying solution. ΔA–area curves are usually shifted upwards and are parallel to each other as the pH is decreased.[2] Similar changes have been observed with fatty acids, the surface potential being higher in the unionized than in the ionized state (132). Even negative potentials may arise from ionization of the end groups. Adam (3) has ascribed this to the fact that in the unionized state the molecules are oriented with the positive carbon of the carboxyl group above the negative oxygen, whereas dissociation produces the formation of an additional dipole of opposite direction, the positive hydrogen ion below, and the negative oxygen above. The resultant moment of these oppositely directed dipoles will determine the total difference in surface potential. As similar changes with amines are of much smaller magnitude, the total effects observed with zwitterionic proteins may well be explained on that basis.

Philippi (125) has attempted to evaluate the maximum charge on a protein monolayer from surface potential data, using a modification of Chapman's (26) and Herzfeld's (72) equation for the drop of the interfacial

[2] If the ionic strength of the medium is sufficiently high, the surface potential is independent of pH.

tension of a water surface when it becomes electrically charged. The calculated values are considerably lower than those derived from acid- and base-binding capacity. The disagreement has been ascribed to a decrease of the dipole moment due to penetration of counter ions between the polar heads of the film molecules. The variations of film pressures with pH, mentioned in the previous section, have been related by Philippi to the electric repulsion between charged film molecules and counter ions, respectively, and to osmotic forces exerted by the counter ions. Neglecting any specific adsorption, he has calculated, with the aid of Chapman's and Herzfeld's equations, the film pressure variations from the corresponding surface potential data. It is the reviewers' opinion that such considerations, although of considerable interest, are somewhat premature at the present stage of our knowledge.

The influence of denaturation on protein spreading

Undoubtedly native proteins denature upon spreading. The extensive structural changes involved, as is shown by the radically altered molecular dimensions, permit of no other conclusion. There is, unfortunately, considerable misunderstanding concerning the term "denaturation." We employ it here to mean a profound structural change in the protein molecule, and it involves, as recent work indicates, a change from a condensed to a more extended molecular configuration (9, 21, 118). Such denatured protein is readily soluble away from its isoelectric point. Coagulation, on the other hand, is a change of the soluble protein (denatured or native) to an insoluble condition. There is reason to believe that compressed protein films are coagulated as well as denatured. Devaux (34) was able to show that such films have no solubility at all. On the other hand, at very low film pressures the amount of protein adsorbed from very dilute solutions into the surface film was a function of the amount of protein in solution (34), indicating that in expanded films the protein is denatured but not coagulated. This point deserves the attention of investigators.

Lee and Wu (99) showed that urea-denatured egg albumin can be completely spread on a buffer at pH 7.0. Bull (20) was able to spread both heat-denatured egg albumin and urea-denatured egg albumin on a buffer at pH 4.9, providing the protein solutions being spread were first adjusted to a pH of 2.2. Further, he was able to show that both these forms of denatured egg albumin, as well as native protein, when spread, gave practically the same pressure–area relations, indicating an identical structure for these three spread substances.

If the solution of denatured protein from which spreading is to take place is too close to the isoelectric point, spreading will not occur, as Neurath

has shown (115). Evidently the denatured protein is coagulated on the surface before it can spread. Gorter (49) has shown that the addition of traces of pepsin restores completely the spreading power of heat-denatured (coagulated) egg albumin in much the same manner as trypsin aids the spreading of proteins which otherwise can be spread only on dilute acids. Heat-coagulated egg albumin, without the use of enzymes, will definitely not spread.

Rideal (130), Neurath (115), and Gorter (45) have investigated the action of ultraviolet light on the properties of completely spread protein films. It was found that the films collapse under the influence of irradiation; the film area becomes smaller with increasing time of irradiation. Ultraviolet light of different wave lengths seems to produce different effects (130).

Langmuir (92) has recently reported that the so-called "surface patterns," produced by placing an "indicator oil" on top of a protein film, are different for spread denatured proteins than for spread native proteins. The reviewers are unaware of the experimental details of these investigations and cannot, therefore, evaluate the significance of these findings.

Structure of protein monolayers

Numerous attempts have been made to relate the observed properties of protein films to the structure and orientation of the film molecules. As it is impossible in this review to give due consideration to all the various hypotheses which have been advanced, the readers are referred to the original papers. The following discussion merely outlines the reviewers' opinion regarding this problem.

There is considerable evidence for the assumption that, in the completely expanded form, protein monolayers consist of polypeptide chains of the beta-keratin type (11), with the amino acid residues stretched out flat along the surface (53, 76, 77, 109, 124). If 3.5 Å. be taken as the length of a peptide group in the direction of the chain and 4.5 Å. as the average length of a side chain (11), and if allowance is made for alternate orientation of the side chains, the calculated minimum area is 31.5 sq. Å. This value is to be compared with the values listed in table 6, column 5. The mean areas per amino acid residue in sq. Å., Ar, given in table 6 were calculated according to the following relation:

$$Ar = \frac{A \times Mr \times 10^{23}}{6.06 \times 10^{23}}$$

where Mr is the average molecular weight per residue and A the film area in square meters per milligram of protein.

The agreement between areas calculated in this manner and those calcu-

lated from molecular dimensions is, considering the assumptions involved, in satisfactory accord. The approximate values for the average residue weight, the average length of the side chains, and the limiting area at zero compression may well and readily account for the slight deviations.

Hughes and Rideal (77) and Mitchell (109) reported higher values than those given in table 4 for the areas of gliadin, zein, and insulin per amino acid residue. These films, however, were spread on $N/100$ hydrochloric acid, which is well on the acid side of the isoelectric point of these proteins, and under these conditions electrostatic repulsive forces between ionized end groups would tend to keep the side chains apart. This is indicated

TABLE 6

Observed and calculated film areas of proteins

PROTEIN	AVERAGE RESIDUE WEIGHT	LOW-PRESSURE REGION EXTRAPOLATED TO ZERO PRESSURE			HIGH-PRESSURE REGION AT COLLAPSING PRESSURE			
		Area per milligram	Film thickness	Mean area per residue	Area per milligram	Film thickness	Mean area per residue	
							All R groups on one side	Alternate orientation of R groups
		square meters	*Å.*	*square meters*	*square meters*	*Å.*	*square Å.*	*square Å.*
Egg albumin (20, 115, 125)	124 (12)	1.3	5.7	27.5	0.77	9.6	15.9	31.8
Insulin (109, 125)	122 (78)	1.65	4.5	33.0	0.77	9.6	15.5	31.0
Serum albumin (115)	134 (27)	1.45	5.1	32.0	0.79	9.4	17.2	34.2
Gliadin (3, 77)	120 (109)	1.65	4.5	32.8	0.50	About 15	10.0	20.0
Cytochrome C (68)	120*	1.55	4.9	30.6	0.70	10.0	14.0	28.0

* Estimated.

by the fact that when gliadin is spread on a phosphate buffer at pH 5.9, the limiting area per residue is 32.8 Å.

Evidence is also available from the study of the tripeptide α-aminocaprylic acid (58). When this tripeptide is spread at pH 4.6, the limiting area is about 93 sq. Å., and since the calculated area per molecule with the side chains lying flat on the surface is 116 sq. Å., but only about 50 sq. Å. if they are perpendicular to the surface, it may be assumed that the side chains are lying flat.

We have noted in our preceding discussion that certain proteins form rigid, coherent films in the low-pressure region, whereas others are in the liquid state (50, 92, 125). There is, no doubt, a tendency for the polar group of neighboring peptide chains to attract each other and, similarly,

the non-polar groups have a "mutual solubility." Such a state of affairs would tend to give a certain structure or rigidity to the film. Whether or not a coherent film is obtained depends, in the reviewers' opinion, mainly on the degree of "fitting" of the end groups; close fitting would bring the side groups within the sphere of their attraction and tend to produce a coherent film. The influence of pH on the properties of expanded protein films, mentioned in a previous section, may likewise be accounted for on the basis of the above picture. Repulsive forces, evoked by conferring positive or negative charges on the protein film, will greatly decrease the lateral adhesive forces between neighboring polypeptide chains.

The thickness of a fully extended protein film, using the density of protein in bulk, is about 5 Å.

Lateral compression of expanded films produces drastic changes in the orientation of film molecules, as is indicated by a gradual decrease in the dipole moment and by a decrease in the coefficient of compressibility. Also, the films which are liquid in the low-pressure regions become rigid on compression. In addition (20), it has been observed that the adhesion tension between films deposited on glass and water drops sharply at 1.5 dynes pressure.

While slight compression may merely cause the protein molecules to close in laterally without change in orientation of the side chains (77, 109), undoubtedly under higher pressures the side chains will be forced out of the surface until they are oriented perpendicular to the surface. The reorientation is probably complete when the slope of the pressure–area curve attains its maximum value. Closest packing will, however, be attained only at very high pressures near the collapse point.

It is possible to obtain some information regarding the structure of highly compressed films from the areas occupied by such films and from the molecular dimensions. If the protein molecules have a fully stretched beta-keratin configuration, then the area per residue should be 15.7 sq. Å. (11). This value agrees with the area per residue as calculated from film areas if it is assumed that all side chains are on the same side of the plane (table 6, column 8). As, however, the side chains are essentially of hydrocarbon nature, their minimum film area cannot be less than between 20.5 and 25 sq. Å. (3). Alternate orientation of the side chains would yield ample room for them in the film. It is to be expected that polar side chains would be directed towards the water and the apolar chains towards the air. Since in a number of proteins about one half of the total residues are polar and the other half apolar (105, 125), the minimum area per amino acid would be about 31.5 sq. Å. This value is compared with the calculated film areas in table 6, column 9. If there is a large excess of apolar or polar side chains, the area per residue at the collapse point would be

somewhat larger than calculated on the assumption of alternate orientation (109).

The thickness of protein films at the collapse point is likewise in good agreement with this picture. Column 7 of table 6 lists the respective values. They are in the neighborhood of 10 Å., which is the value assigned to the side chain spacing of the beta-keratin from x-ray studies. The film thickness should be only about 5 Å. if all side chains were oriented on the same side of the surface. The investigation of the structure of deposited monolayers of egg albumin by means of x-rays confirms the idea that the side chains are oriented perpendicular to the surface (10).

The rigidity of the compressed films may, in addition to the action of residual affinities between peptide linkages of neighboring chains, be ascribed to the adhesion between now vertically close-packed side chains.

Compression of the films in the high-pressure region probably results also in a dehydration in the film (40, 77, 109, 125). In fact, Philippi (125) attributes the decrease in area of the film on increasing pressure, even in the low-pressure regions, to a dehydration of loosely bound water. It should be pointed out, however, that Philippi tacitly assumed that all residues are directed towards one side of the surface in low pressures and towards both sides in the high-pressure region, but did not explain the change of orientation during compression. The reviewers have indicated their reasons for believing that the change in orientation of side chains from a horizontal to a vertical position is mainly responsible for the pressure–area relations in the low-pressure region and that such dehydration as does occur is incidental.

Stereochemical considerations and valence angle requirements seem to rule out any folding of the polypeptide chains upon compression. As all naturally occurring amino acids are structurally related to *l*-lactic acid, the orientation of the residues will be alternate in a *fully* extended polypeptide chain; partial or complete ring formation, however, would result in all residues attached to carbon atoms of a ring being oriented on the same side of the plane. It may be readily seen that neither the alpha-keratin, nor the "supercontracted"-keratin configuration (7), nor any other ring structure would allow sufficient room for the vertically oriented side chains in the film and would result in areas per residue much smaller than those observed.

Wrinch (143) advanced the theory that protein films consist of "cyclol" fragments, i.e., cages broken down into plane fragments, held together either by hydrogen bonds or else by salt linkages. It is a requirement of such a structure that the side chains be on the front surface of the network, out of the water, whereas peptide hydroxyl groups emerge from both surfaces of the network. The area per amino acid residue has

been given as about 10 sq. Å. for polymers of "cyclol 6", and as 13.3 and 16.8 sq. Å., respectively, for less dense structures, such as polymers of "cyclol 18" and "cyclol 66". The actual available areas are, in the reviewers' opinion, probably even smaller, owing to the uneven distribution of side chains within the network, and owing to the spaces taken up by peptide hydroxyls. As, according to Wrinch's view, protein films retain at least fragments of cyclols, it follows that in the densest spots of the film there cannot be enough room for the side chains, which require a minimum surface area of between 20 and 25 sq. Å. Similar considerations may be applied to the surface requirements of closed cyclols, such as the C_2 molecule, which has been assigned to the structure of proteins of the 40,000 molecular weight class.[3] The assumption that ionized side groups which emerge from the upper surface are bent so that they lie in the water phase would add to the difficulty of fitting this hypothesis to the area requirements, since a bent chain should occupy a greater cross-sectional area than if it were straight.

Rideal (131), Philippi (125), and Mitchell (109) have pointed out that a protein monolayer may be regarded as a triplex film which consists of an upper hydrocarbon layer of low dielectric constant and of a thickness of about 3.5 to 4.5 Å. Below this layer is the middle layer, formed by the main chains and associated water molecules, and finally below the middle layer are the polar side chains.

Very little is known about the structural changes which proteins undergo during spreading. Gorter (50) and Neurath (115) have described the process as an unfolding of polypeptide chains. Astbury (7) has suggested that spreading brings about a separation of disks which, in the native molecule, are about 10 Å. apart. It is the reviewers' belief that a final elucidation of this problem, which is so intimately connected with the problems of protein structure and denaturation, must await further work in the field of protein chemistry.

Mixed films

By means of surface potential and surface pressure measurements, Rideal, Schulman, and Hughes investigated associations and interactions between proteins and other surface-active materials (104, 131, 133, 135, 136). Digestion of egg albumin and caseinogen films by pepsin and trypsin, dissolved in the underlying solutions, has been studied by Schulman and Rideal (135). From the studies, as far as they have gone, these authors concluded that the disappearance of a protein film on an enzyme-

[3] A detailed discussion of this problem is in preparation and will be published elsewhere.

containing substrate is due only to digestion and not to adsorption of some capillary-active constituents of the enzyme. This viewpoint has been revised as a consequence of further detailed investigations (133) concerning the behavior of protein monolayers on solutions containing surface-active substances.

Injection of a surface-active substance underneath a surface film has been found to affect greatly the surface potential and surface pressure of the film. Mere anchoring of the injected molecules by suitable polar groups of the film alters the surface potential without changing the surface pressure. Thus, gallic acid, tannic acid, or certain dyes are adsorbed beneath protein monolayers without penetrating them. If, however, there is in addition to polar interaction a strong attraction between the hydrophobic groups of the material beneath the film and the hydrophobic groups of the film molecules, the injected molecules penetrate the film. Such penetration can readily be detected by a considerable increase of surface pressure, which is well above the possible film pressure of either of the components individually. Film pressures of as high as 60 dynes have been observed with stable mixed films (136). Complete film penetration will cause the surface potential to become identical with that of a mixed film containing equimolecular proportions of the two molecules at maximum compression (133). Stable mixed films have been obtained with gliadin and cholesterol up to film pressures of 20 dynes (136). Film penetration may increase or decrease the rigidity of a protein monolayer, depending on the pH and the electrolyte concentration. Triolein completely liquefies a gliadin film, but gelation takes place upon strong compression of a mixed gliadin-tripalmitin film (136). If the interaction between the molecules of two components of a mixed film is weak, lateral compression will force one molecular species out of the film. Thus, a stable and rigid cholesterol–gliadin film liquefies suddenly at pressures above 20 dynes, revealing all the characteristics of a cholesterol film (136). It is of interest that this process is reversible. Likewise, monolayers of egg albumin have been reported to become displaced by injection at pH 7.2 of long-chain fatty acids underneath the film. Film penetration may be completely prevented by compressing the film, prior to injection, to the equivalent pressure at which displacement would start. The significance of the phenomena of film adsorption, penetration, and displacement, respectively, for agglutination, sensitization, and hemolysis has been pointed out by Schulman and Rideal (136).

The properties of mixed films of egg albumin and myristic acid, obtained by simultaneous spreading of these substances from solution, have been investigated by Neurath (117). By assuming that the protein occupies the same film area in the presence of the acid as it does when spread alone,

no film interaction could be detected with films containing between 100 and 400 molecules of myristic acid per molecule of protein. With films containing less myristic acid, a marked increase of the area per fatty acid molecule was observed, and with a mole ratio of 9 reached a value of more than 180 sq. Å.

Protein films deposited on solids

Langmuir and his associates, following the technique which had been used with stearate monolayers, have carried out interesting experiments[4] in which spread protein films were deposited on solid surfaces (16). Langmuir, Schaefer, and Wrinch (97) have described the properties of monolayers of egg albumin transferred to chromium plates on which layers of barium stearate had been deposited. The results obtained have been taken as support for the view that protein monolayers consist of a two-dimensional network and not of polypeptide chains. Neurath (116) has pointed out that, under the conditions of these experiments, the occurrence of film collapsing prior to deposition has to be envisaged and that the properties of such films may be accounted for in terms of inhomogeneous, collapsed layers. The lack of a well-defined hydrophobic or hydrophilic character of deposited protein monolayers has been explained by Langmuir (91) on the assumption that protein monolayers may "overturn" in response to the conditions under which they have been deposited. The reviewers find it difficult to reconcile this hypothesis with the view that protein films consist of a two-dimensional network. Whereas polypeptide chains could be expected to possess the degree of flexibility which, according to Langmuir's picture, is necessary for overturning of stearate films, considerable constraint must be imposed upon the flexibility of protein films if they consist of a two-dimensional network.

Langmuir and Schaefer (96) reported that monolayers of urease and of pepsin, deposited on conditioned plates, have a high chemical activity as measured by the decomposition of urea and by the clotting of milk, respectively. The data indicate that monolayers of urease were active only when deposited as B-layer, whereas in the case of pepsin both A- and B-layers were active. The reviewers believe that, in view of the general importance of such studies, severely controlled experimental conditions are of utmost necessity. Langmuir and Schaefer report that monolayers of urease were obtained by spreading on distilled water at pH 5.8. Gorter and Maaskant's (56) experiments seem to indicate, however, that in that

[4] In view of the forthcoming publication of a summarizing article by Dr. Langmuir in this Journal, the reviewers have refrained from a detailed discussion of his work on protein monolayers.

pH range the spreading of urease requires more than 16 min. to be complete. It may also be pointed out that, according to Langmuir and Schaefer's data, active monolayers of urease vary in thickness from 11 to 21 Å. when deposited under 0.4 dyne pressure, whereas the thickness of a homogeneous protein film is about 5 Å. at low pressures and hardly exceeds 10 Å. even near the collapsing pressure. Likewise the thickness of 14 Å., reported for active monolayers of pepsin, seems to be too high for a homogeneous monolayer.

Gorter (48) reported that monolayers of pepsin and trypsin, removed from the surface by a silk gauze and dissolved again in buffer solution by proper adjustment of the pH, retained about 80 per cent of their original activity. These results have been taken as proof that either the monolayers themselves are active or else that the activity has been regained upon dissolution of the surface-denatured enzyme.

Astbury, Bell, Gorter, and van Ormondt (10) have deposited up to seventeen hundred successive layers of egg albumin on chromium-plated metal slides. These films have a tendency to tear parallel to the direction in which the slide was moved through the liquid phase. They are birefringent when viewed perpendicular to the surface, the slow vibration being parallel to the direction of movement of the slide. Some of the films investigated showed numerous boat-shaped holes (negative tactoids) pointing parallel to the direction of movement of the slide. The thickness of these films, measured by direct mechanical means, has been found to be 9.5 Å. per monolayer, in excellent agreement with the results obtained with the surface balance.

The influence of deposited layers of fatty acids and proteins on the changes in shape of mammalian erythrocytes has been investigated by Ponder and Neurath (127). From the results obtained these authors concluded that a red cell can hemolyze when less than half of its surface is in contact with a monolayer of a lytic substance, and even when there are not enough lysin molecules to cover the whole cell surface with a monolayer.

REFERENCES

(1) ABRAMSON, H. A.: Electrokinetic Phenomena. The Chemical Catalog Co., Inc., New York (1934).
(2) ADAM, N. K.: Trans. Faraday Soc. **29**, 90 (1933).
(3) ADAM, N. K.: The Physics and Chemistry of Surfaces. The Clarendon Press, Oxford (1938).
(4) ADAM, N. K., AND HARDING, J. B.: Proc. Roy. Soc. (London) **A138**, 411 (1932).
(5) ARTOM, C.: Arch. sci. biol. **14**, 327 (1929).
(6) ASCHERSON, F. M.: Arch. Anat. Physiol., p. 44 (1840).
(7) ASTBURY, W. T.: Chem. Weekblad **33**, 778 (1936).
(8) ASTBURY, W. T.: Trans. Faraday Soc. **34**, 377 (1938).

(9) ASTBURY, W. T.: Cold Spring Harbor Symposia on Quant. Biol. **6** (1938), in press.
(10) ASTBURY, W. T., BELL, F. O., GORTER, E., AND VAN ORMONDT, J.: Nature **142**, 33 (1938).
(11) ASTBURY, W. T., AND WOODS, H. J.: Nature **126**, 913 (1930); Trans. Roy. Soc. **A232**, 333 (1933).
(12) BERCZELLER, L.: Intern. Z. phys. chem. Biol. **1**, 124 (1914); Biochem. Z. **53**, 215, 232 (1913).
(13) BERGMANN, M., AND NIEMANN, C.: J. Biol. Chem. **118**, 301 (1937).
(14) BILTZ, W., AND STEINER, H.: Biochem. Z. **23**, 27 (1910); Z. physik. Chem. **83**, 683 (1913).
(15) BLODGETT, K. B.: J. Optical Soc. Am. **24**, 313 (1934).
(16) BLODGETT, K. B.: J. Am. Chem. Soc. **57**, 1007 (1935).
(17) BRIGGS, D. R.: J. Am. Chem. Soc. **50**, 2358 (1928).
(18) BROSSO, A., AND FREUNDLICH, H.: Z. physik. Chem. **89**, 306 (1914).
(19) BULL, H. B.: J. Biol. Chem. **123**, 17 (1938).
(20) BULL, H. B.: J. Biol. Chem. **125**, 585 (1938).
(21) BULL, H. B.: Cold Spring Harbor Symposia on Quant. Biol. **6** (1938), in press.
(22) BULL, H. B.: Unpublished results.
(23) BULL, H. B., AND NEURATH, H.: J. Biol. Chem. **118**, 163 (1937).
(24) BULL, H. B., AND NEURATH, H.: J. Biol. Chem. **125**, 113 (1938).
(25) CARY, A., AND RIDEAL, E. K.: Proc. Roy. Soc. (London) **A109**, 301 (1925).
(26) CHAPMAN, D. L.: Phil. Mag. **25**, 475 (1913).
(27) COHN, E. J.: Ergeb. Physiol. biol. Chem. exptl. Pharmakol. **33**, 781 (1931).
(28) DAVIS, C. E., SALISBURY, H. M., AND HARVEY, M. T.: Ind. Eng. Chem. **16**, 161 (1924).
(29) DE CARO, L.: Arch. sci. biol. **14**, 247 (1929).
(30) DE CARO, L., AND LAPORTA, M.: Arch. sci. biol. **14**, 264 (1929).
(31) DEUTSCH, V.: Compt. rend. **203**, 183 (1936).
(32) DEUTSCH, F.: Compt. rend. **203**, 252 (1936).
(33) DEVAUX, H.: Proc. verb. soc. des sci. phys. et nat. Bordeaux, November, 1903; January, 1904; Kolloid- Z. **58**, 129, 260 (1932).
(34) DEVAUX, H.: Compt. rend. **200**, 1560 (1935).
(35) DOW, P.: J. Gen. Physiol. **19**, 907 (1936).
(36) DUMMETT, A., AND BOWDER, P.: Proc. Roy. Soc. (London) **A142**, 382 (1933).
(37) DU NOÜY, P. L.: Surface Equilibria of Biological and Organic Colloids. The Chemical Catalog Co., Inc., New York (1926).
(38) ERRERA, J., AND MILLAN, E.: J. chim. phys. **30**, 726 (1933).
(39) ETTISCH, G., DOMONTOWITSCH, M., AND MUTZENBECHER, P. v.: Naturwissenschaften **18**, 447 (1930).
(40) FOSBINDER, R. J., AND LESSIG, A. E.: J. Franklin Inst. **215**, 579 (1933).
(41) FOURT, L., AND SCHMITT, F. O.: J. Phys. Chem. **40**, 989 (1936).
(42) FU, J., AND WU, H.: Proc. Soc. Exptl. Biol. Med. **27**, 878 (1930).
(43) FREUNDLICH, H., AND ABRAMSON, H. A.: Z. physik. Chem. **133**, 51 (1928).
(44) GORTER, E., AND GRENDEL, F.: Proc. Acad. Sci. Amsterdam **34**, 1257 (1925).
(45) GORTER, E.: Am. J. Diseases Children **47**, 945 (1934).
(46) GORTER, E.: Proc. Acad. Sci. Amsterdam **37**, 20 (1934).
(47) GORTER, E.: J. Gen. Physiol. **18**, 421 (1935).
(48) GORTER, E.: Proc. Roy. Soc. (London) **A155**, 706 (1936).
(49) GORTER, E.: Trans. Faraday Soc. **33**, 1125 (1937).

(50) GORTER, E.: The Chemistry of the Amino Acids and Proteins. Edited by C. L. A. Schmidt. Charles C. Thomas, Springfield, Illinois (1938).
(51) GORTER, E., AND GRENDEL, F.: Proc. Acad. Sci. Amsterdam **29**, 371 (1925).
(52) GORTER, E., AND GRENDEL, F.: Proc. Acad. Sci. Amsterdam **29**, 1262 (1926).
(53) GORTER, E., AND GRENDEL, F.: Biochem. Z. **201**, 391 (1928).
(54) GORTER, E., AND GRENDEL, F.: Trans. Faraday Soc. **22**, 477 (1926).
(55) GORTER, E., AND MAASKANT, L.: Proc. Acad. Sci. Amsterdam **40**, 71 (1937).
(56) GORTER, E., AND MAASKANT, L.: Proc. Acad. Sci. Amsterdam **40**, 74 (1937).
(57) GORTER, E., MAASKANT, L., AND VAN LOOKEREN CAMPAGNE, G. J.: Proc. Acad. Sci. Amsterdam **39**, 1187 (1936).
(58) GORTER, E., MEIJER, T. M., AND PHILIPPI, G. TH.: Proc. Acad. Sci. Amsterdam **37**, 355 (1934).
(59) GORTER, E., AND PHILIPPI, G. TH.: Proc. Acad. Sci. Amsterdam **37**, 788 (1934).
(60) GORTER, E., AND SEEDER, W. A.: Naturwissenschaften **19**, 738 (1931); Kolloid-Z. **58**, 257 (1932).
(61) GORTER, E., AND SEEDER, W. A.: Kolloid-Z. **61**, 246 (1932).
(62) GORTER, E., AND SEEDER, W. A.: J. Gen. Physiol. **18**, 427 (1935).
(63) GORTER, E., AND VAN ORMONDT, J.: Proc. Acad. Sci. Amsterdam **36**, 922 (1933).
(64) GORTER, E., AND VAN ORMONDT, J.: Biochem. J. **29**, 48 (1935).
(65) GORTER, E., VAN ORMONDT, J., AND DOM, F. J.: Proc. Acad. Sci. Amsterdam **35**, 838 (1932).
(66) GORTER, E., VAN ORMONDT, J., AND MEIJER, TH. M.: Biochem. J. **29**, 381 (1935).
(67) HARKINS, W. D.: J. Chem. Phys. **1**, 852 (1933); **3**, 693 (1935).
(68) HARKINS, W. D., AND ANDERSON, T. F.: J. Biol. Chem. **125**, 369 (1938).
(69) HARKINS, W. D., AND ANDREWS, T. F.: J. Am. Chem. Soc. **59**, 2189 (1937).
(70) HARKINS, W. D., AND MYERS, R. J.: J. Chem. Phys. **4**, 716 (1936).
(71) HERCIK, F.: Kolloid-Z. **56**, 1 (1931).
(72) HERZFELD, K. F.: Physik. Z. **21**, 28, 61 (1920).
(73) HITCHCOCK, D. I.: J. Gen. Physiol. **8**, 61 (1925); **10**, 179 (1926).
(74) HOPKINS, F. G.: J. Physiol. **25**, 306 (1900).
(75) HOPKINS, F. G.: Nature **126**, 328, 383 (1930).
(76) HUGHES, A. H.: Trans. Faraday Soc. **29**, 214 (1933).
(77) HUGHES, A. H., AND RIDEAL, E. K.: Proc. Roy. Soc. (London) **A137**, 62 (1932).
(78) JENSEN, H., AND WINTERSTEINER, O.: J. Biol. Chem. **98**, 281 (1932).
(79) JERMOLENKO, N.: Kolloid-Z. **48**, 141 (1929).
(80) JOHLIN, J. M.: J. Phys. Chem. **29**, 271 (1925).
(81) JOHLIN, J. M.: J. Phys. Chem. **29**, 897 (1925).
(82) JOHLIN, J. M.: J. Gen. Physiol. **11**, 301 (1927).
(83) JOHLIN, J. M.: J. Biol. Chem. **76**, 559 (1928).
(84) JOHLIN, J. M.: J. Phys. Chem. **29**, 1130 (1925); J. Biol. Chem. **84**, 543 (1929).
(85) JOHLIN, J. M.: J. Biol. Chem. **87**, 319 (1930).
(86) JOHNSTON, ST. J. H.: Biochem. J. **21**, 1314 (1927).
(87) JOHNSTON, ST. J. H., AND PEARD, G. T.: Biochem. J. **19**, 281 (1925); **20**, 816 (1926).
(88) KEMP, I., AND RIDEAL, E. K.: Proc. Roy. Soc. (London) **A147**, 1 (1934).
(89) LANDSTEINER, K., AND UHLIRZ, R.: Centr. Bakt. Parasitenk., Abt. orig. **40**, 265 (1905).
(90) LANGMUIR, I.: J. Am. Chem. Soc. **39**, 1848 (1917).
(91) LANGMUIR, I.: Science **87**, 493 (1938).
(92) LANGMUIR, I.: Cold Spring Harbor Symposia Quant. Biol. **6** (1938), in press.

(93) LANGMUIR, I., AND SCHAEFER, V. J.: J. Am. Chem. Soc. **59**, 2400 (1937).
(94) LANGMUIR, I., AND SCHAEFER, V. J.: J. Am. Chem. Soc. **59**, 1406 (1937).
(95) LANGMUIR, I., AND SCHAEFER, V. J.: J. Am. Chem. Soc. **59**, 1762 (1937).
(96) LANGMUIR, I., AND SCHAEFER, V. J.: J. Am. Chem. Soc. **60**, 1351 (1938).
(97) LANGMUIR, I., SCHAEFER, V. J., AND WRINCH, D. M.: Science **85**, 76 (1937).
(98) LANGMUIR, I., AND WAUGH, D. F.: J. Gen. Physiol. **21**, 745 (1938).
(99) LEE, W., AND WU, H.: Chinese J. Physiol. **6**, 307 (1932).
(100) LINDAU, G., AND RHODIUS, R.: Z. physik. Chem. **A172**, 321 (1935).
(101) LOEB, J.: J. Gen. Physiol. **6**, 105 (1923).
(102) LOEB, J.: J. Gen. Physiol. **2**, 577 (1919).
(103) LOUGHLIN, W. J.: Biochem. J. **27**, 106 (1933).
(104) MARSDEN, J., AND SCHULMAN, J. H.: Trans. Faraday Soc. **34**, 748 (1938).
(105) MARTIN, W. M.: J. Phys. Chem. **38**, 213 (1934).
(106) MELSENS, M. L.: Ann. chim. phys. **3**, 33, 170 (1851).
(107) METCALF, W. V.: Z. physik. Chem. **51**, 1 (1905).
(108) MICHAELIS, L., AND RONA, R.: Biochem. Z. **15**, 196 (1909).
(109) MITCHELL, J. S.: Trans. Faraday Soc. **33**, 1129 (1937).
(110) MOSS, S. A., JR., RIDEAL, E. K., AND BATE SMITH, E. C.: Nature **136**, 260 (1935).
(111) MOYER, L. S.: J. Bact. **31**, 531 (1936).
(112) MOYER, L. S.: J. Phys. Chem. **42**, 71 (1938).
(113) MOYER, L. S.: J. Biol. Chem. **122**, 641 (1938).
(114) MOYER, L. S., AND ABRAMSON, H. A.: J. Biol. Chem. **123**, 391 (1938).
(115) NEURATH, H.: J. Phys. Chem. **40**, 361 (1936).
(116) NEURATH, H.: Science **85**, 289 (1937).
(117) NEURATH, H.: J. Phys. Chem. **42**, 39 (1938).
(118) NEURATH, H.: Cold Spring Harbor Symposia on Quant. Biol. **6** (1938), in press.
(119) NEURATH, H., AND BULL, H. B.: J. Biol. Chem. **115**, 519 (1936).
(120) PAIC, M., AND DEUTSCH, V.: Compt. rend. **202**, 1283 (1936).
(121) PAIC, M., AND DEUTSCH, V.: Compt. rend. **202**, 1514 (1936).
(122) PALMER, A. H.: J. Gen. Physiol. **15**, 551 (1932).
(123) PAULI, W., AND VALKO, E.: Kolloidchemie der Eiweisskoerper. Th. Steinkopff, Dresden (1933).
(124) PHILIPPI, G. TH.: Rec. trav. chim. **53**, 81 (1934).
(125) PHILIPPI, G. TH.: On the Nature of Proteins, Thesis, University of Leiden, 1936.
(126) PLATEAU, J.: Statique des Liquides **2**, 261 (1873).
(127) PONDER, E., AND NEURATH, H.: J. Exptl. Biol. **15**, 358 (1938).
(128) PRIDEAUX, E. B. R., AND HOWITT, F. O.: Proc. Roy. Soc. (London) **B112**, 13 (1932).
(129) RAMSDEN, W.: Arch. Physiol., p. 1517 (1894); Proc. Roy. Soc. (London) **B72**, 156 (1903); Z. physik. Chem. **47**, 336 (1904).
(130) RIDEAL, E. K.: Kolloid-Z. **61**, 218 (1932).
(131) RIDEAL, E. K.: Proc. Roy. Soc. (London) **A155**, 684 (1936).
(132) SCHULMAN, J. H., AND HUGHES, A. H.: Proc. Roy. Soc. (London) **A138**, 436 (1932).
(133) SCHULMAN, J. H., AND HUGHES, A. H.: Biochem. J. **29**, 1236 (1935).
(134) SCHULMAN, J. H., AND RIDEAL, E. K.: Proc. Roy. Soc. (London) **A130**, 259 (1931).
(135) SCHULMAN, J. H., AND RIDEAL, E. K.: Biochem. J. **27**, 1581 (1933).
(136) SCHULMAN, J. H., AND RIDEAL, E. K.: Proc. Roy. Soc. (London) **B122**, 29 (1937).
(137) SEASTONE, C. V.: J. Gen. Physiol. **21**, 621 (1938).

(138) SMEE, A. H.: Proc. Roy. Soc. (London) **12**, 399 (1863); **13**, 350 (1864).
(139) SPIEGEL-ADOLF, M.: Biochem. Z. **252**, 37 (1932).
(140) TER HORST, M. G.: Rec. trav. chim. **55**, 33 (1936).
(141) WILHELMY, L.: Ann. Physik **119**, 177 (1863).
(142) WOODS, H. J.: Nature **132**, 709 (1933).
(143) WRINCH, D. M.: Proc. Roy. Soc. (London) **A160**, 59 (1937); **A161**, 505 (1937); Phil. Mag. **25**, 705 (1938).
(144) WU, H., AND LING, S. M.: Chinese J. Physiol. **1**, 407 (1927).
(145) WU, H., AND WANG, C.: J. Biol. Chem. **123**, 439 (1938).
(146) ZOCHER, H., AND STIEBEL, F.: Z. physik. Chem. **147**, 401 (1930).