[CONTRIBUTION FROM THE RESEARCH LABORATORY, THE GENERAL ELECTRIC CO.]

Pressure-Soluble and Pressure-Displaceable Components of Monolayers of Native and Denatured Proteins

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A drop of pure liquid hydrocarbon placed on a clean water surface holds together as a circular lens in spite of the force of gravity which tends to spread the hydrocarbon over the surface. There is thus a definite contracting force of about 11 dynes/cm., which prevents complete spreading of the lens. If molecules having both hydrophilic and hydrophobic parts are in solution in either the hydrocarbon or the underlying water, these molecules concentrate at the interface, and there exert a spreading force F_I. If this force equals or exceeds the force of contraction, the lens spreads to form a duplex film,[1] so thin that gravitational forces are negligible.

With a limited amount of spreadable substance at the interface, the oil spreads until the surface concentration decreases to the point at which F_I is again equal to the critical value. The area to which the oil spreads varies in proportion to the amount of adsorbed substance in the interface. The thickness of the lens may thus decrease until interference colors appear. With a further introduction of spreading substances the lens may become too thin to give interference colors.

The local introduction of solid protein or protein solution beneath a lens results in a sharply defined decrease in thickness in this region, the surrounding regions remaining much thicker.[2]

The arrival of minute traces of spreading substances at a hydrocarbon interface can be detected by using indicator oils as described by Blodgett.[3] Automobile lubricating oil is oxidized by heating, thus producing hydrophilic groups, so that a drop of the oil spreads to give an invisible film. This oil, when mixed with a non-spreading oil such as petrolatum, produces an indicator oil which forms stable films of uniform thickness. By choice of proportions, an indicator oil film can be made to show any desired interference color when the film is not confined by a barrier, *i. e.*, at $F = 0$. When a dilute solution of protein, biological peptone, or other spreadable substance, is injected beneath such an indicator oil film, the arrival of the molecules at the interface causes a change in the interference color. The original color is chosen so that a given small increase in area produces a maximum change in color.

Indications of Soluble Products in Protein Monolayers.—Recently[4] Schaefer described a simple method for the classification of protein monolayers. A small drop of indicator oil is applied from the end of a platinum wire to the center of a protein monolayer which has been made coherent by compression to 1.0 dyne/cm. The outline of the expanding oil takes a geometrical shape which is characteristic of the protein used. These expansion patterns have been classified as star like, rough circular, and smooth circular. In expansion patterns obtained with pure insulin (smooth circular) the area occupied by the fully expanded indicator oil is seen to present a uniform color.

In expansion patterns made with trypsinogen and trypsin, however, the expanded oil does not give a uniform interference color but shows a gradation in color near its boundary which corresponds to a thinning of the oil in this region. The gradation, easily seen in Figs. 2c and 3a of reference (4), suggests the following. During the spreading of the protein monolayer a portion of the protein, or impurities in the protein, remains in the substrate just beneath the surface. The expanding monolayer carries with it, as it spreads, a sheath of this dilute protein solution. When the drop of indicator oil is placed in the central region of the protein monolayer, it expands and pushes ahead of it the protein monolayer and the associated sheath of protein solution. At the same time the expanding oil drags its own sheath of pure water along with it. During expansion, however, both sheaths lag behind the surface films over them. At the boundary between oil and protein this lag causes the dilute protein sheath to be displaced, so that part of it comes to lie under the edge of the indicator oil. Thus, protein diffuses gradually into the oil film near its boundary and

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(1) I. Langmuir, *J. Franklin Inst.*, **218**, 143 (1934).

(2) I. Langmuir, V. J. Schaefer and D. M. Wrinch, *Science*, **85**, 76 (1937).

(3) K. B. Blodgett, *J. Optical Soc. Am.*, **24**, 313 (1934).

(4) V. J. Schaefer, *J. Phys. Chem.*, **42**, 1089 (1938).

produces a thinning with consequent changes in color. It has been our experience that the edge effect appears within one minute after expansion.

Observations on edge effects suggest the possibility of using the phenomenon for a semi-quantitative estimation of spreading molecules present just beneath the surface. It should be capable of indicating not only unspread portions of proteins but also any film components which may be forced to leave the surface because of increased solubility under pressure.

In another publication,[5] in which the reversibility of the force-area curve of gliadin was studied, it was found that after compression to 25 dynes/cm. the area at 1 dyne was less than its original value. A progressive decrease in area was observed after successive compressions to 10, 15, and 25 dynes/cm. As a possible explanation it was suggested that the decrease in area is due to "a small proportion of short-chain degradation products produced during the spreading of the protein, which are driven into solution by subjecting the film to a high pressure." It should be possible to demonstrate the presence of these short-chain degradation products by a modification of the edge-effect technique.

A study of reversibility in the compression of protein monolayers and a correlation with indicator oil discoloration are needed to establish the characteristics of the pure protein. In the present publication we report observations made with untreated proteins and with proteins that have been subjected to various forms of degradation.

The Edge Effect in Protein Monolayers.—We propose to use the change in the interference color exhibited by an indicator oil film to measure the amount of spreading substances in the substrate. On distilled water, in the absence of strong light, such oil films are fairly stable over a period of fifteen minutes or more; but with a substrate of 0.01 *N* hydrochloric acid a noticeable color change is observed within five minutes. We have avoided taking data, therefore, if the oil film is more than ten minutes old and in most cases have used substrates having *p*H's between 4 and 8. The indicator oil used in the following experiments gave a first-order blue-purple at $F = 0$ and 45° angle of incidence.

We recognize three types of edge effect: I, edge effects due to protein molecules or impurities which have remained in the substrate because of incomplete spreading; II, edge effects produced by the mechanical admixture of the indicator oil with the protein film; and III, edge effects due to substances which spread with the protein, remain in the surface film at low pressures but leave the interface at high pressures and pass into the substrate.

I. Edge Effects Due to Incomplete Spreading.—To this class belong all edge effects in expansion patterns observed when the films have not been subjected to pressures over 2–3 dynes/cm. A number of experiments indicate that the substances which produce these edge effects are in solution in a very thin sheath just beneath the surface.

The expansion pattern of a film of commercial pepsin (Lilly) on distilled water, which has never been under any pressure other than the spreading pressure, exhibits a silver edge of 0.5 cm. width. If, however, the film is allowed to remain at $F = 0$ for twenty minutes before applying the indicator oil, no edge effect appears, because the spreadable molecules in solution just under the film diffuse deeply into the substrate and thus return so slowly and diffusely to the surface that they give no appreciable edge effect. A thorough agitation of the substrate under the film of commercial pepsin at $F = 0$ is sufficient to prevent the formation of an edge effect. Again, if an expansion pattern which exhibits a symmetrical edge effect is suddenly displaced 2–3 cm. by simultaneous movement of both confining barriers, the original edge effect is modified by the superposition of a further discoloration along the advancing edge of the oil film. This is caused by the shifting of the protein sheath from a position beneath the protein to a position beneath the oil.

Crystalline trypsinogen (Northrop's, isoelectric point 7.0–8.0) when spread on distilled water (*p*H 5.8) yields a low specific area and shows a wide light-silver edge effect. On a substrate at *p*H 7.0 the specfic area at 1 dyne/cm. increases to 0.7 m.²/mg.; the edge effect entirely disappears. Analogous results are found with crystalline pepsin: a wide edge effect at *p*H 7.0, while at *p*H 2.0 near the isoelectric point no edge effect is observed. The edge effect is found to increase with the deviation of the *p*H of the substrate from the isoelectric point of the protein used.

These results run parallel to those obtained on the thicknesses of protein monolayers.[5] It was found that over a range of *p*H's giving widely

(5) I. Langmir and V. J. Schaefer, *Chem. Rev.*, **24**, 181 (1939).

different specific areas the thicknesses of the resulting monolayers at $F = 16.5$ dynes remained constant. The apparent variations in specific area with *p*H of the substrate are thus due to incomplete spreading of the original protein.

The fact that a pure protein generally does not spread completely on a substrate far from its isoelectric point has allowed us to test the strength of a commercial pepsin by means of film measurements. Crystalline pepsin yields a specific area of 0.90 m.²/mg. at 1 dyne/cm. when spread on hydrochloric acid substrate at the isoelectric point, *p*H 2.6, and gives no edge effect. When spread at the same *p*H commercial pepsin yields a specific area of 0.10 m.²/mg. and exhibits a wide silver edge effect. If all the pepsin, and none of the protein impurities in the commercial pepsin, spreads at *p*H 2.6, the activity should thus be 11% of that of the crystalline material. A sample of the same commercial pepsin actually showed 16% of the activity of crystalline pepsin[6] when tested by its power to clot skimmed milk.

The production of a pure pepsin monolayer from an impure pepsin solution spread at *p*H 2.6 was also shown by Langmuir and Schaefer.[6] The milk-clotting power of monolayers deposited on metal plates as PRA_L films at $F = 9$ dynes/cm. was found to be the same (22,000 and 21,000 units per gram, respectively) whether the monolayers were spread from commercial or from crystalline pepsin.

II. **Edge Effects Produced by Mechanical Admixture.**—The circular expansion pattern of insulin shows no edge effect. If the expanded indicator oil and surrounding protein films are compressed to 25 dynes/cm. and then re-expanded to 0 pressure, a distinct edge effect is seen. By repeated compressions and expansions the protein and indicator oil may be completely intermixed, giving a composite film showing a very light brown interference color. A pressure of only 5 dynes/cm. causes the indicator oil film to collapse into a small lens or thick ridge, depending upon the viscosity of the protein monolayer. At the higher pressure of 25 dynes/cm. the protein monolayer is probably forced into folds under the oil and remains at the oil–water interface after the pressure is removed. This type of edge effect seems to be of little practical significance, but care must be taken that it is not confused with the other types.

(6) I. Langmuir and V. J. Schaefer, THIS JOURNAL, **60**, 1351 (1938).

III. **Edge Effects Due to Substances Forced Out of the Monolayer.**—This type of discoloration, which we shall attempt to correlate with permanent decreases in area of protein monolayers, will be discussed in greater detail later. That the phenomenon exists may be proved in the following manner. The spreading end and the balance end of a film trough are designated in Fig. 1 by A and F. A flat glass sheet G, extending the full width of the trough and about half the length of the tray, is supported so that its surface is 1 mm. below the edges of the trough. The glass sheet extends to within 0.5 cm. of the film balance.

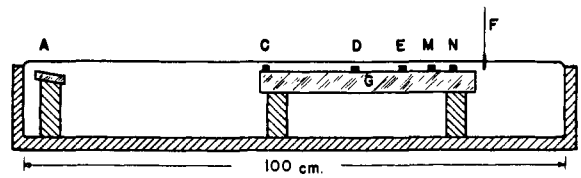

Fig. 1.—Trough with submerged barriers for measuring the surface pressures needed to force pressure-soluble components into solution. The vertical scale is exaggerated 15-fold.

A series of submerged barriers C, D, E, M, N (nickel bars 1 mm. × 5 mm. extending the width of the trough which have been previously oxidized by heating in a Bunsen flame to render them hydrophilic) are now placed on the glass plate in predetermined positions. They are adjusted so that the sweeping and compression barriers are just able to pass over them. Barrier C is a "scrubbing" barrier which scrapes the under surface of the monolayer being spread at A and prevents unspread protein molecules from being carried into the compression region of DEMN. The positions of these barriers have been determined so that, when a fixed quantity of protein is spread, D is just beneath the compressing barrier at 10 dynes/cm., E at 15 dynes/cm., M at 20 dynes/cm., and N at 25 dynes/cm. The movable compressing barrier is left above each submerged barrier for a two-minute period, after which it is moved to the next position and the solution between the submerged barriers, from which the monolayer has just been removed, is tested with indicator oil for spreadable molecules.

A solution of gliadin in 70% alcohol was spread using Gorter's technique (specific area 1.07 m.²/mg. at 1 dyne/cm.). Little or no discoloration was obtained in the 10- and 15-dyne/cm. regions, while a very apparent effect was obtained in the 20- and 25-dyne/cm. regions. This result has

been checked with other proteins. In general such pressure-soluble components are not forced out of the film by pressures up to 10 dynes/cm., but leave the film very rapidly when pressures of 20 and 25 dynes/cm. are reached.

The Spreading of the Monolayers.—Methods of spreading proteins have been discussed previously.[5] In the present experiments we have used protein solutions of 0.333% concentration and to obtain more reproducible specific areas have devised a modification of the band method.[5] A black glass spreading plate 5 cm. wide and as long as the width of the trough is placed on glass strips in the spreading end of the trough so that its back edge is 1 mm. below the edges of the trough (A, Fig. 1). The plate has a gentle slope toward the direction of spreading, so that the forward edge is about 2 mm. below the edges of the trough. The protein solution is delivered from a micropipet as a band about 3 mm. wide near the lower edge of a nickel strip 0.25 mm. thick, 1.5 cm. wide, and as long as the trough width. The nickel strip is then lowered rapidly into contact with the back edge of the spreading plate. As spreading proceeds, the upflow of water along the band with resulting eddy currents between the water surface and the spreading plate tends to force any traces of solution, which otherwise might be lost in the substrate, up to the air–water interface. Although spreading is quite rapid, a period of two to five minutes should be allowed for the completion of the process. Using this method, we have been able to obtain specific areas with deviations less than ±5%.

For the measurement of force a chainomatic balance with a sensitivity of 0.1 dyne/cm. was employed.

Force–Area Curves and their Changes with Time

A striking characteristic of protein monolayers is their relatively great compressibility, and the high degree of reversibility in their force–area curves. Langmuir and Schaefer[5] have emphasized that the monolayers of many proteins can be subjected repeatedly for short times to surface pressures of 25 dynes/cm., and yet the area, say, at 5 dynes/cm. may remain constant.

Many investigators of protein monolayers have noted slow changes in the force–area curves when the film is kept for many hours on a water surface. It is also reported that protein films show partial collapse if compressed to $F = 15$ dynes/cm. or more. These variations with time have been regarded as troublesome effects, whose elimination was attempted by waiting long periods of time until the area at any value of F had reached a steady value. In general it seems to have been thought that this steady state represents a true equilibrium. Langmuir,[7] however, has pointed out that the spreading of a protein to form a monolayer is an irreversible process and that the monolayer when subjected to pressure cannot be considered to be in a state of equilibrium with the underlying solution. There appears therefore to be no theoretical reason for attaching particular significance to a steady state which can be nothing more than a pseudoequilibrium. It is far more significant that the force–area curve of a protein monolayer has such a high degree of reversibility when the changes in F are made rapidly.

Mitchell[8] recommends that the force–area curves be determined only after the film has been kept on the water surface for twenty-four hours or more. In recent experiments Cockbain and Schulman[9] allowed a time of two to five hours before observations were made, ". . . so that the film should attain a steady state; . . . the reason for these slow changes is still obscure." The great inconvenience in waiting these long times is surely not justified when there is no theoretical reason for preferring a steady state. In fact, serious errors may be caused by contamination from dust, or from impurities in the substrate.[7,10] To devise a thoroughly rational procedure for the study of protein monolayers it is necessary to determine the causes of the slow changes of the force–area curves with time.

Fundamentally, it must be recognized that with protein films we are dealing with a problem involving three variables—force, area and time, whose three-dimensional relationship we shall represent by the symbol (F, a, t). As a matter of convenience and simplicity it is generally necessary to express this relationship in terms of one or more curves corresponding to two-dimensional relations, (F, a), (F, t), and (a, t).

The procedure that we have adopted in studying these time effects has been to vary F as a function of t in a cyclic manner, so that at the end of each cycle F is brought back to an initial value such as 1 dyne/cm. Usually we choose a standard sequence of rising values of F, each applied for a definite time interval such as one minute, followed by a sequence of falling values. This series of changes in F and t we call a compression–expansion cycle, or (F, t) cycle. The changes in area that occur during the cycle can be expressed by an (F, a) curve.

(7) I. Langmuir, *Cold Spring Harbor Symposia Quant. Biol.*, **VI.** 171 (1938).

(8) J. S. Mitchell, *Trans. Faraday Soc.*, **33**, 1129 (1937).

(9) E. G. Cockbain and J. H. Schulman, *ibid.*, **35**, 1266 (1939).

(10) G. I. Jenkins and T. W. J. Taylor, *Nature*, **142**, 291 (1938).

Figure 2 is a somewhat idealized diagram of a series of (F, a) curves showing typical effects of successive (F, t) cycles. The ordinates F are expressed in dynes/cm., while the abscissas are areas a in m.^{2}/mg. The monolayer after it has been spread is compressed to 1 dyne/cm., and the specific area is measured, giving the point represented by A. If the film is subjected, at one-minute intervals, to a series of pressures, say F = 5, 10, 15, 20, up to 25 dynes/cm., and the area is measured at the end of each one-minute period, a curve AB is obtained. The pressure is then maintained constant for a definite time, t_{BC}, such as ten or fifteen minutes, during which the area gradually decreases, as indicated by the line BC. These changes in area may be expressed as a function of t by a curve (a, t). At the end of the time t_{BC} the pressure F is decreased in a series of steps, one minute each, until F has reached the initial value of 1 dyne/cm., thus completing the (F, t) cycle and giving the curve CD. It will be noted that this initial cycle causes a permanent decrease in the area represented by the difference AD. The curve ABCD does not form a closed loop.

We shall describe these changes produced by the initial (F, t) cycle as *non-reproducible*, since they cannot be reproduced with the *same film*, although of course the same effect can be repeated by using a new film.

Our experiments have shown that the permanent alterations in the film properties produced by the initial cycle, which are conveniently measured by the change AD, are due mainly to effects that occur during the time t_{BC} while the film is subjected to high pressure. These changes we shall describe by the term *pressure aging*.

This aging takes place much more rapidly at higher values of F_{BC}, but pressures over 30 dynes/cm. are usually to be avoided for reasons that we shall discuss later. If the time of aging t_{BC} at a pressure F_{BC} is continued long enough (for example, with insulin, ten minutes at 30 dynes/cm. or twenty minutes at 25 dynes/cm.) the film reaches a steady state in which the subsequently measured displacement AD is found to have reached a limiting value that undergoes no further changes if F is not raised above the original aging pressure F_{BC}. A film in this steady state may be said to be *well aged*.

Let us assume that the initial (F, t) cycle, which gave the curve ABCD in Fig. 2, was such as to give a well aged film. Consider now the effect of subjecting the film to a second identical (F, t) cycle, again raising the pressure to 25 dynes/cm. and bringing it back to 1 dyne/cm. During recompression the area follows the curve DE, E being somewhat less than B. While held at constant pressure for the time t_{BC}, the area gradually decreases to C, the same point as in the initial cycle. During expansion the CD portion of the curve is reproduced. Each of a series of identical cycles now gives a repetition of the same three-sided loop DECD.

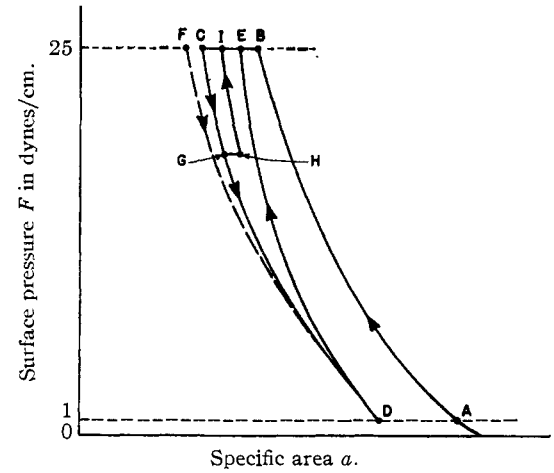

Fig. 2.—Diagram of typical (F, a) curves and their changes with time.

Maintaining the film at 25 dynes/cm. for a longer time than in the initial (F, t) cycle may cause the area to decrease to a point F. However, upon reducing the pressure to 1 dyne/cm. the area again returns to point D, but along some new curve, such as FD, giving a new three-sided loop, DEFD.

If after reaching C in any of these cycles the pressure is reduced only to a point corresponding to G, the section of curve CG is obtained. While the pressure is then held constant, the area increases with time, until some point (H) is reached which lies between the curves CD and DE. Compression now produces the HI portion of the curve, and after a certain time at 25 dynes/cm. the point C may again be reached. Repetition of this last cycle reproduces the four-sided loop CGHIC.

Repeated (F, t) cycles thus give loops in the (F, a) curve similar to DECD or CGHIC. Since these loops in most cases have finite areas, compression–expansion curves are not reversible, although they are reproducible. However, at low

pressures below 15 dynes/cm. the loop may degenerate into a single line, in which case the (F, a) cycle may be termed reversible.

In some cases, to compare differences in character, composition, or structure of films, it is useful to express the force F, not in terms of the area a, but as a function of the ratio a/a_1 of the actual area to that which the film occupied at 1 dyne/cm. just before this compression. This type of curve which represents the function $(F, a/a_1)$ we shall call a *compressibility curve.*

If a portion of a film of a pure substance can be forced into solution by the application of a high pressure such as 25 dynes/cm. the area of the remaining film at a lower pressure will be decreased, but the compressibility curve at these lower pressures remains unaltered. On the other hand, if a film is mostly insoluble at the highest pressure but contains pressure-soluble components, these substances may be driven out of the film by subjecting it to high pressure so that the composition of the remaining film is altered and therefore in general the compressibility and other intrinsic properties will be modified.

The changes with time which we have observed with protein films can be divided into three classes: I, permanent decreases in area produced by initial (F, t) cycles below 30 dynes/cm.; II, permanent decreases in area produced by (F, t) cycles above 30 dynes/cm.; III, reproducible (F, a) loops given by well-aged films.

I. **Permanent Decreases in Area Produced by Initial (F, t) Cycles below 30 Dynes/Cm. Pressure Solubility).**—Protein films (insulin, gliadin) give reversible (F, a) curves when F is kept below about 7–10 dynes/cm. Permanent decreases in area, similar to that shown by AD in Fig. 2, begin to appear after maximum pressures of about 10 dynes/cm. have been applied, and increase rapidly in magnitude as the maximum pressure is raised still more.

Several possible causes may be suggested for the initial permanent decrease in area. A redistribution of linkages between peptide chains within the monolayer might occur. Certain of the less strongly hydrophobic side chains might be driven out of the interface under pressure, coalesce just below the surface, although still attached to it, and remain below the surface after release of the pressure.[5,7] Finally, protein molecules within the film, or more probably impurities or degradation products, when subjected to pressure, could exhibit an increased solubility in the substrate in accord with Gibbs's law.[5] The reappearance of these pressure-soluble components in the interface after expansion would depend upon their ability to diffuse back to and penetrate the protein monolayer, a process which in any case would be very slow.

Whenever a protein film shows a permanent decrease in area at 1 dyne/cm. after an initial (F, t) cycle below 30 dynes/cm., we have found that the presence of soluble products, driven into the substrate by the compression, can be detected by changes in the color of an indicator oil film which is subsequently placed on the surface.

Table I gives the results obtained by applying this method to several proteins. The second column gives the percentage decreases in area (at 1 dyne/cm.) produced in monolayers of these proteins by compressions to 25 dynes/cm. for about five minutes. (A longer time would probably have been better.)

TABLE I

CHANGES IN AREA AND INDICATOR OIL DISCOLORATIONS PRODUCED BY PRODUCTS SQUEEZED OUT OF PROTEIN MONOLAYERS

1	2	3	4	5[a]
Protein	Decrease after 25 dynes/cm., %	Area of monolayer at 1 dyne/cm., sq. cm.	Permanent decr. in area of protein monolayer, sq. cm.	Increase in indicator oil film (total film basis), sq. cm.
Insulin	13	723	94	60– 80
Heated insulin	16	700	112	120
Insulin + pepsin	25	640	160	159
Gliadin	14	958	134	110–130
Egg albumin (1)	3.1	709	22	25
Egg albumin (2)	3.1	710	22	24– 40
Pepsin	2	550	11	<35

[a] Uncertainties in col. 5 are due to the difficulties of judging oil color changes. In the case of pepsin it was necessary to use hydrochloric acid substrate on which indicator oil normally undergoes a fairly rapid change.

To make a quantitative determination of the amount of spreadable substance forced into the substrate, we have adopted the following technique which involves the use of indicator oil. The apparatus shown in Fig. 3 is used. The protein is spread at A. Col. 3, Table I, gives the areas in sq. cm. which were applied and col. 4 gives the permanent decrease in area which would take place if the films were subjected to 25 dynes/cm. pressure. B is a group of three submerged barriers just below the surface which "scrub" any unspread protein molecules, or other substances,

from the under surface of the film. The barrier C serves as an additional precaution against the entry of contaminants into the C-D region. The film balance is shown at F. G, a glass plate 1 mm. below the edges of the trough, carries the submerged barriers C, D, and E.

When the protein film is compressed by a surface barrier to a pressure of 25 dynes/cm., D is just beneath the compressing barrier. The film is kept at 25 dynes/cm. pressure for ten minutes. During the normal decrease in area, corresponding to BC in Fig. 2, which takes place in this ten-minute interval, D is kept beneath the compressing barrier by pushing it along the glass plate by cleaned platinum wires. The compressing barrier is then moved rapidly to E and a limited amount of indicator oil (200–400 sq. cm.) is immediately spread over the exposed solution between D and E (at $F = 0$). Spreading molecules present in the substrate between these submerged barriers, which are derived from the protein monolayer, now diffuse to the oil–water interface and there produce an increase in area of the indicator oil and a consequent change in color due to the decrease in thickness. A small area of fresh indicator oil is spread near C. Since no spreading molecules are present at this point, this area does not change color. The difference in color between this area and the oil over the DE region is noted.

The fractional increase in area of the oil over the DE region corresponding to the observed change in color is determined as follows. Another sample of indicator oil, oxidized to give a silver color, is spread, and by compression between barriers is made to undergo the same color changes as those observed in the foregoing experiments, while the changes in area, shown by the movements of the barriers, are measured. Multiplying these fractional increases in area by the total area between barriers D and F gives the increase which is recorded in the last column of Table I.

A comparison of cols. 4 and 5 shows a close agreement between the permanent decrease in area of the monolayer and the increase in area of the indicator oil film. These should be equal if all spreadable substances forced out of the protein monolayer are transferred to the indicator oil film and if the area which these molecules contribute to the monolayer is the same as that which they give at the indicator oil interface. We have no proof that each of these two conditions is fulfilled. It may be possible by a further study of these pressure-soluble spreadable substances to investigate these factors separately. However, from the close agreement between cols. 4 and 5 we may conclude that the irreversible decreases in specific area after compressions to 25 dynes/cm. are mainly due to the forcing out of pressure-soluble components into the substrate.

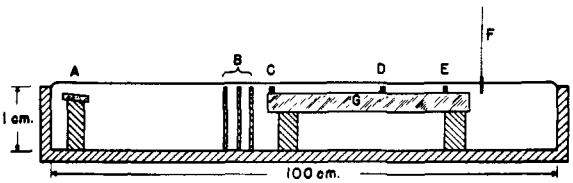

Fig. 3.—Trough with submerged barriers for measuring the amounts of pressure-soluble components.

These pressure-soluble components of monolayers are undoubtedly often impurities in the original protein. We find, for example, in the data of Table I that the purest proteins, crystalline pepsin and egg albumin, have given only small losses in area. However, it is quite possible that pressure-soluble substances may be produced even from pure proteins by the partial breakdown or unfolding of globular protein molecules which must accompany the spreading process.

II. **Permanent Decreases in Area Produced by (F, t) Cycles above 30 Dynes/Cm. (Collapse).**—We have seen that an insulin film loses about 13% of its specific area at 1 dyne/cm. after aging at 25 dynes/cm. If such a film is recompressed to 45 dynes/cm. for ten minutes the total decrease at 1 dyne/cm. is raised to 42%. It is now possible to obtain a fairly reproducible (F, a) loop between pressures of 1–45 dynes/cm. Further, the compressibility curve between 1 and 25 dynes/cm., obtained after the 42% decrease in area, is the same, within the experimental accuracy, as the compressibility curve obtained after the initial 13% decrease in area.

This additional decrease in specific area could apparently result from a further pressure solubility of film components (protein or otherwise). The following experiments have shown, however, that this is not the cause of these area changes produced by high pressures. An insulin film is compressed to 25 dynes/cm. and, by adjustment of the area, is maintained at constant pressure for ten minutes. During the ten-minute period the substrate beneath the monolayer is agitated

by a nickel or glass submerged barrier which is introduced beneath the monolayer from the opposite side of the film balance, suitable precautions being taken to prevent contaminants from building up a pressure on this side of the balance barrier. The major portion of the monolayer is then moved, while kept under 25 dynes/cm. pressure by spaced confining barriers, to another portion of the trough which has been made very shallow (1 mm. deep) and which contains several submerged barriers just beneath the surface. The monolayer is then compressed to an area which would correspond to 40 to 45 dynes/cm. After several minutes, the monolayer is removed and indicator oil is applied to the freshly exposed surface of the substrate to detect the presence of spreadable substances. Such tests have shown mere traces of short-chain products that were apparently forced out between 25 and 30 dynes/cm. There was no observable correlation between these traces and the permanent decreases in area caused by the high pressures. We conclude that practically all the pressure-soluble components of the insulin monolayer are driven out of the film by pressures of 25 to 30 dynes/cm., and that the partial collapse of the film at still higher pressures is not caused by the presence of such substances.

The permanent decreases in area in an insulin monolayer at 1 dyne/cm. produced by ten minutes of exposure to each of a series of increasing pressures are given in Fig. 4. The loss in area increases exponentially up to 25 dynes/cm., the total decrease then being 12% in close agreement with col. 2, Table I. Compression to 30 dynes/cm. increases the loss to only 14%, which is far less than would be given by an extrapolation

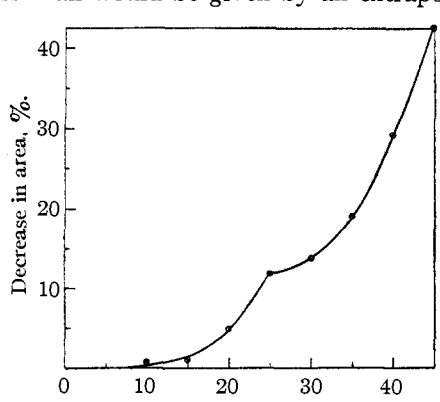

Fig. 4.—Total permanent decreases in area after successive exposures to a series of increasing pressures.

of the lower portion of the curve. Above 30 dynes/cm. there is again an exponential type of curve.

The break in the curve near 25 dynes/cm. is probably related to the fact that below this pressure the loss in area results from pressure-solubility, while above this point it is due to collapse.

Permanent decreases in area of protein films have generally been associated with collapse of the monolayer even at relatively low F values. Thus Mitchell[8] finds "collapse points" at $F = 14$ and at $F = 10$ for films of gliadin and insulin and records no points on the (F, a) curves at pressures above these limits. If our experiments with insulin can be taken to be typical, however, a distinction should be drawn between the effects that occur below and above 30 dynes/cm. The term collapse should be restricted to permanent decreases in area produced by pressure which are not caused by the loss of pressure-soluble components from the film.

III. **Reproducible** (F, a) **Loops Given by Well Aged Films.**—There are several factors that may interfere with the reproducibility of the (F, a) loops, DECD, mentioned in our discussion of Fig. 2.

(1) Contamination of the water surface by dust or by surface-active substances from the edges of the tray or barriers may cause increases in area. Blank runs with no protein film on the surface can be used to detect such impurities. These contaminations usually collapse under low pressure, and therefore do not affect the force–area curves at higher values of F. At low values they may cause a gradual increase of area. In our experiments the tray was kept covered by a glass plate to decrease dust contamination.

(2) Unspread protein molecules or pressure-soluble components driven into the substrate diffuse slowly back to the surface but penetrate the protein monolayer only at low values of F. After compression and re-expansion of a monolayer the area may at first return to its original value and then undergo a slow increase. These effects are more often observed with impure proteins and with those spread on substrates whose pH is not close to the isoelectric point. Difficulties from these sources may be avoided by repeatedly passing the compressed monolayer over submerged barriers (see B in Fig. 3), so as to scrub off the underlying water sheath and then bringing the film over a clean substrate.

(3) With some proteins that give monolayers of high viscosity or rigidity, such as egg albumin or urease, a certain proportion of unspread molecules may be entrapped in the monolayer. If the film is compressed to 10 dynes/cm. or more soon after spreading, these entrapped molecules are prevented from spreading; but when the pressure is reduced to 1 dyne/cm., the film slowly expands to an area greater than that observed at the same pressure before compression. Such effects can be distinguished from those given by soluble spreadable substances in the substrate, since they are not eliminated by passing the compressed film over scrubbing barriers.

(4) Insufficient aging may cause the monolayer to decrease gradually in area in successive (F, t) cycles, especially if the pressure is raised close to or higher than that used in the aging.

(5) If the pressures used during the (F, t) cycles are too high, over 30 dynes/cm., there may be partial collapse with consequent decreases in area.

The shape of an (F, a) loop (except in the case of reversible loops at low values of F) depends upon the particular (F, t) cycle used and can therefore only be reproduced if the successive cycles are identical. When a change is made in the (F, t) cycle, even with a well-aged monolayer, it is often found that the new (F, a) loop becomes reproducible only after the new (F, t) cycle has been repeated a few times. The monolayer, like many elastic-fluid substances, seems to have a memory that may last through more than one cycle.

Figure 5 illustrates the changes that occur in the (F, a) curves obtained with a monolayer of wheat gliadin spread on water buffered at pH 7.2 by 4×10^{-4} M potassium bicarbonate. The gliadin was kindly given to us by R. A. Gortner; it was applied to the surface as 0.1% solution in 65% alcohol. No attempt was made to measure the areas in m^2/mg.; the abscissas in Fig. 5 are the distances in cm. between the movable barrier and the surface balance. They are therefore proportional to the areas of the monolayer.

The point A represents the area when the film is first compressed to 2 dynes/cm., and the curve AB′ shows the changes in area as the pressure is raised in successive one-minute intervals up to 23 dynes/cm. The small circles are used to denote points where no appreciable changes in area occur during the one-minute interval. At 17, 20, and 23 dynes/cm., as shown in the figure, there are appreciable decreases in area during the one-minute interval. After the one minute at 23 dynes/cm., the pressure is lowered in steps, giving the curve whose beginning is indicated by the dotted line below C′. The lower part of the curve approaches very closely the curve AB′.

In order to age the film the pressure is then raised quickly to 25 dynes/cm., giving the point B, and the pressure is maintained constant for ten minutes. The changes in area during the first three minutes are indicated by small marks on the line BC. The (a, t) curve during this ten-minute interval at BC is given in the upper right-hand part of Fig. 5.

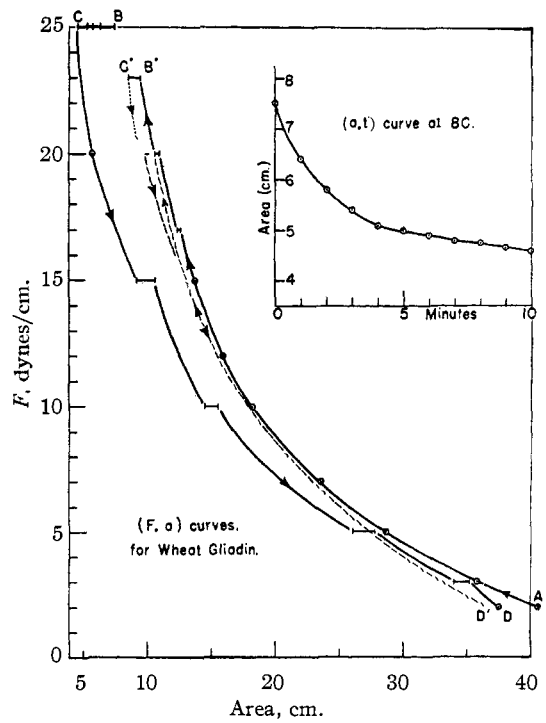

Fig. 5.—Successive (F, a) curves and one (a, t) curve for wheat gliadin.

After this aging treatment, the pressure is decreased in steps of 5 dynes/cm., as indicated by the broken curve CD, being kept constant at each of these points during a one-minute period. At $F = 20$ there is no appreciable expansion during the one minute but at the lower pressures very considerable expansions occur.

The film was then compressed and expanded several times by successive identical (F, t) cycles in which F was raised at one-minute intervals in 5-dyne/cm. steps from 2 to 20. After ten minutes F was lowered in similar steps to 2 dynes/

cm. After the first two cycles, the (F, a) loop became completely reproducible (within 0.1 cm.), giving the loop represented by the dashed line which begins at D′ and extends upward to $F = 20$. During the ten-minute interval at $F = 20$, the area changed from 10.6 to 9.8 cm. and then decreased, as given by the dashed line. At 15 dynes/cm. and below, however, there were no measurable differences between the areas observed with rising and falling pressures, nor were there any detectable changes during the one-minute intervals. Thus below 15 dynes/cm. the (F, a) curve was not only reproducible but also reversible.

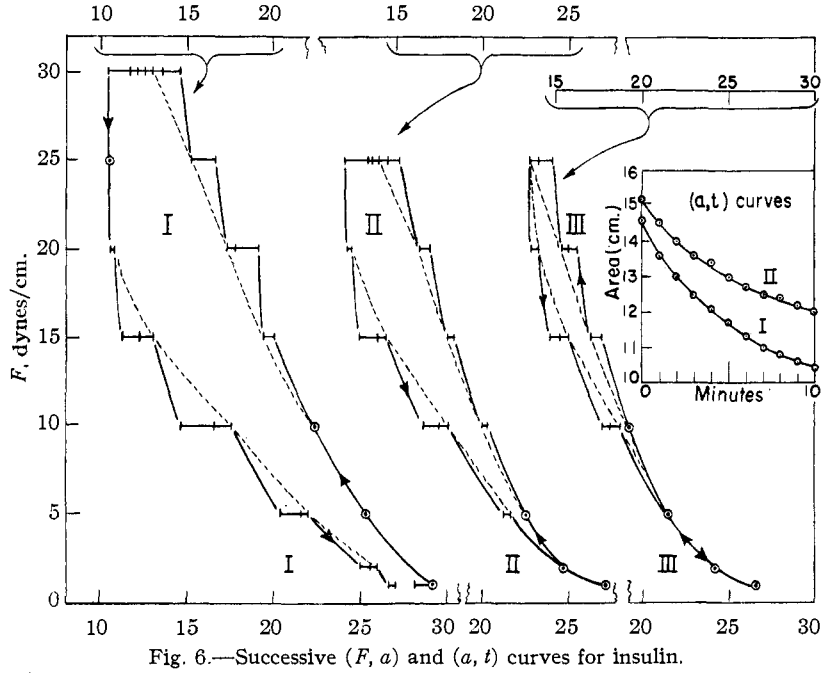

Fig. 6.—Successive (F, a) and (a, t) curves for insulin.

Similar experiments with insulin monolayers gave the results summarized in Fig. 6. The monolayer was spread on distilled water at *p*H 5.8 from a 0.3% aqueous solution of insulin hydrochloride. The (F, a) Loops I, II, and III were made with (F, t) cycles, in which the film was compressed in a series of steps, holding the pressure constant at each step for a two-minute interval, except that at the highest pressure used, in Loops I and II, the interval was ten minutes. The points marked by circles are those in which there is no appreciable change in area during the two minutes. Where changes did occur, these are indicated by horizontal heavy lines with vertical marks giving the areas at successive minute intervals. In some cases, such as at $F = 15$ and 25, in the right-hand part of Loop I, where only one such mark is shown, the change was practically complete within the first minute. In order to prevent overlapping of the curves, three separate scales are used for the abscissas, which represent distances between barriers in cm.

The insulin film was compressed by a force of 1 dyne/cm. within one minute after spreading and gave an initial area of 28.2. During the next minute this reading increased to 29.2 but remained constant during the next two minutes, indicating that the spreading of the protein was nearly complete. The (a, t) curve (I) inserted in the upper right corner of Fig. 6 gives the changes in area during the ten-minute interval at 30 dynes/cm. Upon lowering the pressure to 25 dynes/cm., only a slight expansion occurred, and the area did not change with time. However, at 15 and particularly at 10 dynes/cm. there was a rapid and progressive expansion. At the end of the cycle the area at 1 dyne/cm. reached 27.0 within one minute and remained constant during the next minute. This permanent decrease in area from 29.2 to 27.0, as we have previously seen, is associated with pressure-soluble components. The (F, t) cycle of Loop I also served to age the film.

In Loop II the force was raised to only 25 dynes/cm. The (a, t) curve (II) obtained during ten minutes at this pressure is given in the insert. When the pressure was lowered to 2 and to 1 dyne/cm., the area returned to the same values that were observed during the compression.

In Loop III the (F, t) cycle was like that of Loop II, except that the pressure was maintained at 25 dynes/cm. for only two minutes instead of ten minutes, a change which greatly altered the shape of the (F, a) loop. Further repetitions of this cycle gave loops which were identical with Loop III, even after the monolayer was allowed to remain on the surface under a protecting glass cover for eighty minutes at $F = 1$.

The dashed curves in Fig. 6, which connect the points giving the areas after two-minute intervals, are smooth curves comparable to those of Fig. 2

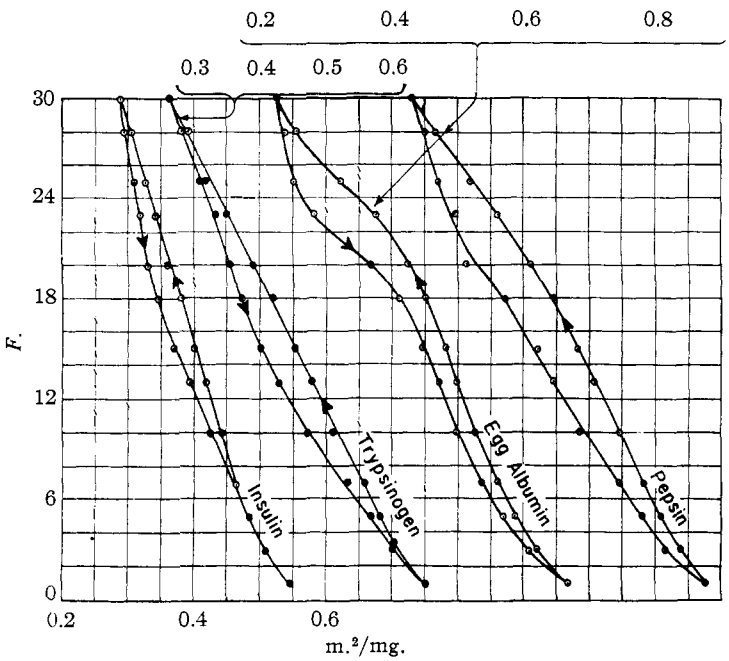

Fig. 7.—Reproducible (F, a) loops for well-aged monolayers of several proteins.

In Loop III the flat part of the dashed curve at the highest pressure degenerates to a point because the film was held only two minutes at this pressure.

Figure 7 gives comparative data with four proteins obtained before the desirability for a definite and thorough aging treatment was recognized. Preceding each of these runs the pressure had been raised in about 12 steps up to 30 dynes/cm. and back in an equal number of steps to 1 dyne/cm., holding the pressure constant for a one-minute interval at each pressure. This presumably gave only a rough approximation to complete aging. The curves of Fig. 7, which have three different scales of abscissas, represent points taken at the end of successive one-minute periods. They thus correspond to the dashed Curve III in Fig. 6. The loops of the aged films of all these proteins are seen to be reproducible, since they give closed loops. In some cases they were repeated several times without appreciable change. The shape of each loop is highly characteristic of the protein.

Figure 8 gives the compressibility curves, (F, a/a_1), for five proteins. Since the abscissas represent the ratios between the areas, a, at given values of F and the area, a_1, at 1 dyne/cm., all the curves coincide at this pressure. By this method of plotting the data the characteristic differences between the proteins are not masked by difficulties arising from possible incompleteness of spreading. Each of these five curves was obtained by using a series of rising pressures, the pressure being held constant for one minute at each point.

Figure 9, which extends to low values of a/a_1, contains data obtained by V. J. Schaefer giving the compressibilities of well aged monolayers for three additional proteins: zein, edestin and gelatin (Knox). These proteins were chosen because their amino-acid compositions have been determined.[11] The curves for other proteins, from the same source as the data of Fig. 8, are included for comparison.

Analysis of (F, a) Curves, Duplex-Film Theory.—The spreading of a water-soluble protein to give an insoluble monolayer depends on the presence of hydrophobic side chains in some of the amino acids of the protein.[7,12] In aqueous solutions of the globular proteins these hydrophobic groups must be inaccessible, probably being enclosed within cage-like protein molecules having hydrophilic surfaces: a structure like that of the micelles in solutions of soap and other detergents in which hydrocarbon chains are packed into the interior of spherical micelles whose surfaces contain all the polar groups.

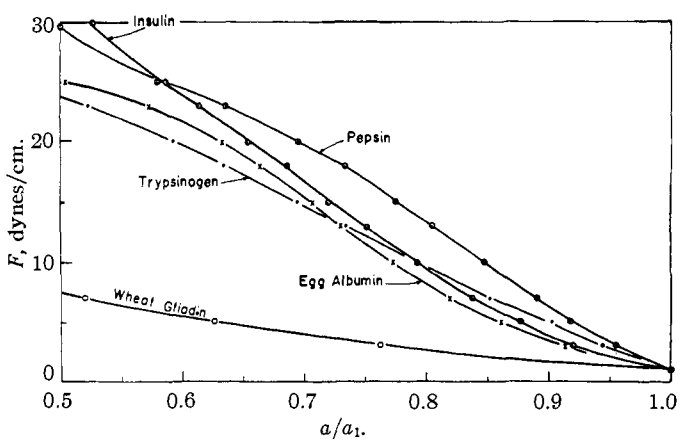

Fig. 8.—Compressibility curves for five proteins.

(11) "The Chemistry of the Amino Acids and Proteins," edited by Carl L. A. Schmidt, 1938, Table IV, Chapter 5, by H. O. Calvery, p. 217.

(12) I. Langmuir, *Proc. Roy. Soc.* (London), **A170**, 1 (1939).

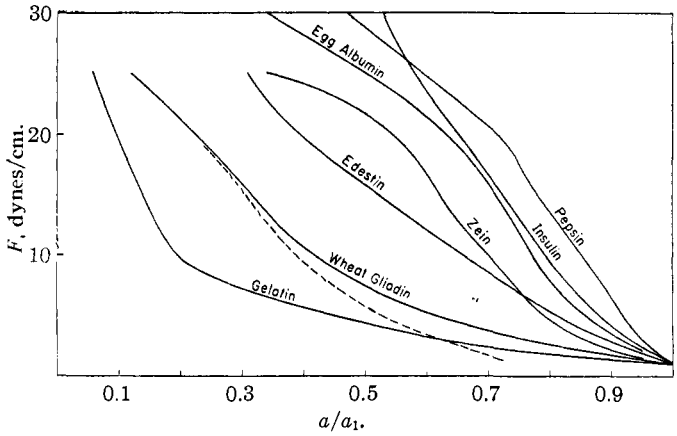

Fig. 9.—Compressibility curves for seven proteins. The dashed curve represents the area of the wheat gliadin film after 20% of ammonium sulfate was introduced into the substrate.

The spreading of the protein on water involves the tearing open or unfolding of the highly organized and symmetrical[13] molecule to give a film consisting of long and probably closed polypeptide chains. At intervals along the length of these chains there are hydrophobic groups which anchor[14] the chains at these points to the air–water interface, leaving the intervening sections of the chains, which are predominatingly hydrophilic, free to form loose folds in the water.

Although the hydrophobic groups are thus interconnected through the chains, thermal agitation should tend to cause them to spread as a two-dimensional gas over the surface as far as the chains will permit. However, this tendency must be in part constrained by the elastic properties of the long chains that result from thermal agitation.[15] It has been proposed[5,7] that protein monolayers have a structure resembling the duplex films[16,1] of myristic acid on acidified water (*p*H 2). These duplex films of fatty acids consist of a thin three-dimensional hydrocarbon liquid phase or interstratum bounded by two interfaces. The upper interface is that between a hydrocarbon surface and air, while the lower interface is one between a hydrocarbon liquid and water in which there exists a spreading force, because the hydrophilic groups act as molecules of a two-dimensional gas.

This duplex-film theory leads to the following equation of state for expanded films

$$(F - F_0)(a - a_0) = kT \qquad (1)$$

where F_0 is the spreading force of the hydrocarbon film without the hydrophilic groups ($F_0 = -11$ dynes/cm. for myristic acid), a is the area of the film per molecule and a_0 is a correction for the finite size of the hydrophilic heads.

The fact that the experimentally determined (F, a) curves of expanded films of fatty acids give hyperbolas which agree with Eq. (1) indicates that each hydrophilic group acts as a single molecule of a two-dimensional gas at the lower interface of the duplex film. Since in the experiments one measures the areas of the film per gram of substance placed upon the surface, the application of Eq. (1) provides a means for determining molecular weights.

The lower portions of the curves for gelatin, gliadin, and edestin in Fig. 9 resemble typical curves for expanded films. Plotting F as a function of a_1/a (the reciprocal of the abscissa in Fig. 9), one obtains straight lines for values of F below certain limits. The curves are thus accurately represented by the following hyperbolas

For gelatin: $(F + 2.3)(a/a_1) = 3.3$ up to $F = 5$ (2)
For gliadin: $(F + 5.4)(a/a_1) = 6.4$ up to $F = 15$ (3)
For edestin: $(F + 16.1)(a/a_1) = 17.1$ up to $F = 10$ (4)

These equations are of the same form as Eq. (1) if $a_0 = 0$ in Eq. (1) and if F_0 takes the values -2.3, -5.4, and -16.1 for gelatin, gliadin, and edestin, respectively.

With these values of F_0 in Eq. (1), putting $a_0 = 0$, $F = 1$, and $T = 293°$K., we obtain for a_1 (the area per active group at the interface when the film is under a pressure of 1 dyne/cm.) the values 121 Å.^{2} for gelatin, 63 Å.^{2} for gliadin, and 23 Å.^{2} for edestin.

The specific area of a gliadin monolayer at $F = 1$ has been found[5] to be 1.1 m.^{2}/mg. The average molecular weight per amino acid residue is 120. Thus the film area per residue at $F = 1$ is 22 Å.^{2}. For proteins such as egg albumin and pepsin, which have specific areas of about 0.7 m.^{2}/mg. at $F = 1$, the area per residue should be about 14 Å.^{2}. These areas are much less than the areas per active group that we calculated from Eq. (1) in the last paragraph.

In the case of gliadin the observed compressibility of the film up to 15 dynes/cm. could be accounted for by assuming that only 35% (*i. e.*, 22/63) of the residues act as active two-dimensional gas molecules at an interface of a duplex

(13) I. Langmuir, *Proc. Phys. Soc., London*, **51**, 592 (1939).
(14) I. Langmuir, *Science*, **87**, 493 (1938).
(15) H. Mark, *Nature*, **141**, 670 (1938).
(16) I. Langmuir, *J. Chem. Phys.*, **1**, 756 (1933).

film, and that there is a constraining force of 5.4 dynes/cm. that opposes the free expansion of these active groups. This proportion (0.35) of actively spreading groups agrees well with the fraction of the amino acids that contain strongly hydrophobic side chains (compare with the ratio $C_3/C_T = 0.356$ for gliadin from the data of Table II). This agreement, if significant, would support our thesis that the hydrophobic groups cause the spreading.

With gelatin the fraction of active residues would be less than 0.2, but with edestin nearly all the residues would need to be active if the compressibility curve is to be in accord with Eq. (1). Although gelatin does have a smaller (0.26) and edestin a greater (0.41) proportion of hydrophobic residues, it is evident that the quantitative agreement fails. For the other proteins the (F, a) curves do not even approximately fit Eq. (1).

In the duplex film theory of expanded monolayers of fatty acids F_0 is taken to be constant (*i. e.*, independent of a), but with protein films, where F_0 is a measure of the constraints due to the elastic properties of the long chains, it is rather to be expected that F_0 should vary with a. Thus Eq. (1) would no longer represent a hyperbola and would be of little practical use in analyzing the properties of protein monolayers.

There is another difficulty in applying the duplex film theory to proteins. With the fatty acid monolayers the number of actively spreading groups (carboxyl radicals) remains constant while a changes. The area per molecule is always greater than 20.4 Å.^{2}, the area in condensed films. With the protein films, however, the area per residue at 1 dyne/cm. ranges from 14 to 22 Å.^{2} and may decrease to very low values such as 2 Å.^{2} at $F = 20$ dynes/cm. Since in these compressed protein films there is not room enough for all the hydrophobic side chains in the surface, we are led to a modified duplex film theory. The side chains that are least strongly hydrophobic are forced into an underlying layer or *underfilm*, although they are still attached by the polypeptide chains to the more strongly hydrophobic groups that remain in the surface layer.

A pressure-displacement of an active component from one part of a duplex film to another was studied by Blodgett in 1934. Drops of Petrolatum to which various proportions of stearic acid had been added (10^{-4} to 10^{-2} parts by weight) were placed on acidified water (0.01 N HCl). With concentrations of stearic acid above a definite limit of 0.31%, the oil spread to form very thin duplex films showing iridescent colors. A quantitative analysis of the (F, a, T) curves of these duplex films proved[17,18] that a part of the stearic acid remained in solution in the hydrocarbon interstratum while the remainder formed an adsorbed film at the lower interface (hydrocarbon–water). This adsorbed film acted as a 2-dimensional gas or liquid (depending on F and T) and gave the variation in F that was observed. However, as the area of the film was decreased by applying external surface pressure, an increasing proportion of the stearic acid was displaced from the lower interface into the interstratum. Based upon this displacement theory equations were derived which expressed accurately the (F, a, T) relations and showed how they depend on the proportions of stearic acid present in the original mixture.

Schulman and Rideal[19] have also observed a case of pressure displacement in their studies of mixed films of gliadin and cholesterol. As the pressure was increased the protein was displaced from the overfilm into an underfilm, while the cholesterol remained in the overfilm.

Pressure-displacement Theory of (F, a) Curves.—The large compressibility of protein monolayers must in large part be due to the progressive squeezing out or displacement of hydrophobic side chains from the overfilm into the underfilm. The groups in the overfilm, because of their close packing at higher pressures, exert forces on one another and so account for most of the spreading force F. When, however, they are forced into the underfilm, they are no longer crowded and therefore contribute little to F. Hydrophobic groups in a water environment tend to adhere to each other just as droplets of pure hydrocarbon liquids in water coalesce to form larger drops. In this way, hydrophobic groups in the underfilm may give weak links between folds of polypeptide chains and so produce the high viscosity or even rigidity often observed in compressed protein films. Other linkages between side chains of polar type may contribute to this viscosity, but in presence of an excess of water such forces should generally play a subordinate role.

(17) I. Langmuir, *J. Franklin Inst.*, **218**, 164–167 (1934).
(18) I. Langmuir, *Gen. Elect. Rev.*, **38**, 402 (1935).
(19) J. H. Schulman and E. K. Rideal, *Proc. Roy. Soc.* (London), **B122**, 46 (1937).

It appears from the data of Figs. 5 to 7 that the time changes in the (F, a) curves of well-aged films, observed at higher pressures, are due neither to soluble components nor to collapse, since the losses in area during compression are regained when the pressure is sufficiently lowered. It will be noted, for example, in Loop I, of Fig. 6, that above 15 dynes/cm. rapid increases or decreases in pressure give relatively small changes in area. The monolayers are thus essentially very incompressible. The large changes in area with time at the upper part of the up-curve and the middle part of the down-curve are not due to the compressibility of the films as such, but result from gradual changes in structure.

According to the displacement theory the forcing out of hydrophobic groups from the overfilm requires folding the polypeptide chains into new configurations. This should generally involve the overcoming of an energy barrier. If this activation energy is large enough, the changes in area at any given temperature take place slowly. By measuring the temperature coefficient of the rate of change it is possible to determine the activation energy.

The displacement of any given group from the overfilm to the underfilm naturally occurs more rapidly at high than at low surface pressures. However, as the pressure on the film is raised, a series of other groups, increasingly hydrophobic, become displaceable by the pressure. A high pressure thus produces an initial rapid change in area followed by very slow changes. The time needed to approach a final state may therefore be greater at higher than at lower pressures.

After the film has been subjected to a high pressure, such as 30 dynes/cm., by which a large proportion of the hydrophobic groups have been displaced into the underfilm, the remaining groups are so tightly packed in the overfilm that the displaced groups cannot return immediately to the overfilm when the pressure is decreased to, say, 20 dynes/cm. If, however, the pressure is brought to a still lower value such as 5 dynes/cm. or less, there is no effective force that prevents the return of these groups to the overfilm and therefore rapid expansion occurs.

On the basis of this theory, the (F, a) curves and the (a, t) curves acquire new significance. The changes with time are dependent on the presence of certain displaceable components in the monolayer. The magnitude of the change in area serves as a measure of the amount of such displaceable material, while the value of F which produces these changes depends upon the relative sizes of the hydrophobic groups. The large hydrophobic side chain of the leucine residue thus requires a high pressure to displace it; but shorter hydrophobic chains can be displaced at much lower pressures.

A summary of the amino-acid compositions of the proteins whose compressibility curves are shown in Fig. 9 is given in Table II, which was prepared from the data given in a table in Schmidt's book.[11] The 20 amino acids which are listed there as constituents of these proteins we divide into three classes, as follows:

Class 1, glycine, alanine, glutamic acid, β-hydroxyglutamic acid, aspartic acid, hydroxyproline, serine, histidine.

TABLE II

THE RELATION BETWEEN THE COMPOSITIONS OF PROTEINS AND THE COMPRESSIBILITIES OF THEIR MONOLAYERS

		Line	Gelatin	Gliadin	Edestin	Zein	Egg albumin	Insulin
				Amino-acid Content (%)				
Class I	C_1	1	58.9	50.4	39.3	47.2	25.1	29.0
Class II	C_2	2	8.4	2.9	16.8	4.1	6.5	15.0
Class III	C_3	3	23.9	29.4	39.4	51.9	39.0	44.0
Total C_T		4	91.2	82.7	95.5	103.2	70.6	88.0
				Relative Areas				
At $F = 3$, a_3/a_1		5	0.62	0.76	0.89	0.85	0.91	0.93
At $F = 25$, a_{25}/a_1		6	0.06	0.14	0.31	0.34	0.50	0.58
				Ratios of Acid Contents[a]				
$(C_1/C_T)_{obsd.}$		7	0.65	0.61	0.41	0.46	0.36	0.33
$(C_1/C_T)_{calcd.}$		8	(0.845)	0.611	0.394	0.460	0.360	0.327
$(C_3/C_1)_{obsd.}$		9	0.41	0.58	1.00	1.10	1.55	1.52
$(C_3/C_1)_{calcd.}$		10	0.40	0.59	0.99	1.07	1.45	1.63

[a] $(C_1/C_T)_{calcd.}$ and $(C_3/C_1)_{calcd.}$ are calculated from the corresponding values of a_3/a_1 and a_{25}/a_1 by Eqs. (5) and (6).

Class 2, hydroxyvaline, cystine, arginine.

Class 3, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophan, proline, methionine, lysine.

The acids of Class 1 are those with side chains having a hydrophobicity not exceeding that of one CH_2 group. We assume that a hydrophilic group, such as -OH, -NH, or -COOH, neutralizes the effect of a neighboring CH_2 or CH_3. A phenyl group was taken as the equivalent of three CH_2's. The acids of Class 2 and Class 3 contain side chains equivalent, respectively, to 2 and to 3 or more CH_2 groups.

The first three lines of Table II give C_1, C_2, and C_3, the amino-acid contents of the proteins included within each of these three classes. The fourth line contains C_T, the total of the acids reported in the analysis, *i. e.*, $C_1 + C_2 + C_3$. Since the proteins are built up of residues derived by elimination of water from the corresponding amino acids, the value of C_T for a complete analysis should be about 115%. The analyses given in the table are thus far from complete.

The acid residues of Class 1, which are the least strongly hydrophobic, should be squeezed out of the overfilm at relatively low pressures. Therefore by the pressure-displacement theory we should expect a correlation between the compressibility of the film at low values of F, and C_1, the content of Class 1 acids. However, in view of the incomplete nature of the analyses, the ratio C_1/C_T should be a more reasonable measure of the Class 1 acids. This corresponds to the assumption that the undetermined acids are distributed among the three classes in proportion to the amounts already found in these classes.

As a measure of the compressibility at low values of F we have selected the ratio a_3/a_1 obtained from the curves of Fig. 9. These ratios are recorded in Line 5 of Table II. A comparison of the ratios C_1/C_T in Line 7 with the corresponding values of a_3/a_1 in Line 5 gives a correlation coefficient of -0.949. However, if the data for gelatin are omitted, the correlation coefficient becomes -0.997. The linear relation thus revealed corresponds to the equation

$$C_1/C_T = 1.88 - 1.67a_3/a_1 \qquad (5)$$

The eighth line in the table contains values of C_1/C_T calculated by Eq. (5) from a_3/a_1 in Line 5. The very close agreement between the observed and calculated values in Lines 7 and 8 proves that the compressibilities of these proteins (except gelatin) are intimately related to their content of Class 1 acids.

The specific areas of films subjected to high pressures, such as 25 dynes/cm., should serve as a measure of the more strongly hydrophobic residues, which correspond to the Class 3 acids. However, our experiments have not given the specific areas, but only the compressibilities, which should depend upon the ratio between the Class 3 and the Class 1 acids. A comparison of C_3/C_1, in Line 9, with the values of a_{25}/a_1 in Line 6, gives a correlation coefficient of $+0.988$. The relationship can also be expressed by

$$C_3/C_1 = 0.26 + 2.37a_{25}/a_1 \qquad (6)$$

The tenth line contains values of C_3/C_1 calculated by this equation from the data of Line 6.

Changes in (F, a) and (a, t) Curves with Temperature.—The pressure-displacement theory suggests that large changes in force–area curves should occur if observations are taken at different temperatures. A measurement of the temperature coefficient of the rate of change in area should permit the calculation of the activation energy associated with the structural changes in the film.

Figure 10-A gives (F, a) curves of a single well-aged insulin monolayer at two different temperatures. The abscissas which represent the distances between the barriers in cm. serve as a measure of the areas. The area at $F = 1$ was the same at both temperatures.

The magnitude in the changes in area is much greater at the higher temperature, yet at the lower pressures at each temperature the area returns to the original value. Figure 10-B gives the (a, t) curves for a well-aged insulin monolayer at three temperatures with the film at a pressure of 25 dynes/cm. after having allowed it to expand to an area of 30.4 by lowering the pressure to 2 dynes/cm. The measurements at the three different temperatures were all made with the same film.

An analysis of these curves shows that when $(a - 5.4)$ is plotted on double logarithmic paper against $(t + 0.5)$, straight lines are obtained. Here a represents the ordinates of Fig. 10-B which measure the areas and t is the time in minutes. These results indicate that the rate of change, da/dt, varies in proportion to $(a - a_\infty)^n$, where $n = 7.8$ at 4°, 3.8 at 24°, and 2.5 at 42°. According to this analysis the limiting value, $a_\infty = 5.4$, should be reached at all temperatures, but the

time required to approach the final value would be extremely long at the lowest temperatures. A rough estimate of the activation energy from these data gives a value of 10 kcal. when $a = 13$, and 20 kcal. at $a = 10$. Thus the activation energy is relatively low for those components of the film which are most easily displaced but becomes high for the more hydrophobic components which are driven out more slowly at higher pressures.

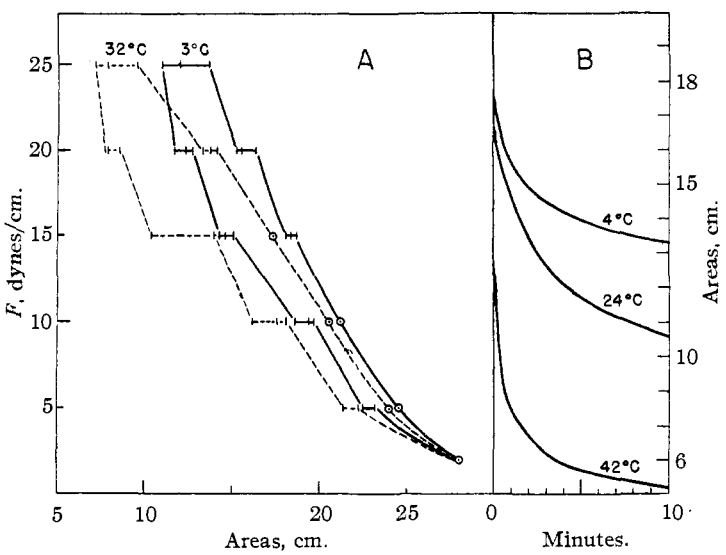

Fig. 10.—The effect of temperature on the (F, a) and (a, t) curves for insulin.

A more detailed analysis of (F, a) curves and their dependence on temperature should reveal effects which depend upon the sequence of the amino-acid residues in the polypeptide chains. A film that is compressed to one-quarter its original area must increase correspondingly in thickness, so that folds of polypeptide chains of at least 60 Å. in length must occur. Particularly at high pressures we should find that several small hydrophobic groups in adjacent positions along the chain produce effects equivalent to a single large group, but at lower pressures the sequence of the amino acids would play a minor role.

Pressure-Soluble and Pressure-Displaceable Components in Monolayers from Degradation Products of Proteins

From evidence summarized in Table I we have seen that the amount of pressure-soluble components in protein monolayers can be determined by measuring the permanent decreases in area produced by initial (F, t) cycles up to 30 dynes/cm. This method should provide a means for studying the nature of the changes that are produced in proteins by denaturation or by the action of proteolytic enzymes. To test its applicability we have made experiments with films produced by spreading solutions of insulin and egg albumin which have been partially digested by pepsin or denatured by heat.

Digestion of Insulin.—A 2% solution of insulin hydrochloride (Lilly) in distilled water was prepared and found to have a pH of 2.6. A 2-ml. volume of this solution was seeded with 0.02 mg. of crystalline pepsin (Northrop) and allowed to digest at 38°. At successive intervals aliquot portions were withdrawn, diluted with distilled water to give a protein concentration of 0.33% and spread by the band-plate method. Measurements were made of: (1) the initial specific area at 1 dyne/cm., (2) the decrease in area at 1 dyne/cm. after successive compressions to a series of increasing pressures, and (3) the compressibility curve $(F, a/a_1)$ of the monolayer remaining after the compression to 25 dynes/cm. The results are summarized in Table III.

Undigested insulin, spread as a monolayer, gave a specific area of 0.57 m.²/mg. at 1 dyne/cm. as shown in the next to the last line and second column of the table.

TABLE III

PERMANENT DECREASES IN AREA OF FILMS OF PARTLY DIGESTED INSULIN, PRODUCED BY EXPOSURE TO A SERIES OF INCREASING PRESSURES

F, dynes/cm.	Time of digestion in hours 0	21	52
5	0.7%	2.6%	15%
10	1.1	10	41
15	4.8	30	70
20	10	44	85
25	13	52	95
Init. sp. area m.²/mg.	0.57	0.31	0.17
Decr. (sol., prods.), %	0	46	70

This film was then subjected to a pressure of 5 dynes/cm. for ten minutes and, after the pressure had been brought back to 1 dyne/cm., the area was found to be 0.7% less than before the compression. In a similar way the film was exposed for successive ten-minute periods to a series of increasing pressures: 10, 15, 20, and 25 dynes/cm. The total decrease in area at 1 dyne/cm. observed after each of these compressions is given

in the second column, expressed as a percentage of the initial area at 1 dyne/cm.

A portion of the insulin solution withdrawn after twenty-one hours of digestion gave a specific area of 0.31 $m.^2$/mg. at 1 dyne/cm. This is 46% less than the value for the undigested protein. This decrease (last line of Table III) measures the fraction of the protein that was converted into soluble substances or into substances which do not spread on water against a pressure of 1 dyne/cm.

This film when subjected for periods of ten minutes at each of a series of increasing pressures showed relatively large changes in area, indicating that the digestion had produced a large increase in the amount of pressure-soluble components. The data in Col. 3 give these total decreases in area at 1 dyne/cm., expressed as percentages of the initial area of this film. Similar data for the film obtained after fifty-two hours of digestion in the last column of the table indicate that 70% of the protein had been made unspreadable at 1 dyne/cm., and that there was a further increase in the amounts of pressure-soluble substance.

The effect of digestion of insulin is thus not merely to produce substances of such low molecular weights that they do not spread, but it changes the character of higher molecular weight substances within the monolayer. For example, the loss in area at 10 dynes/cm. increases four-fold when the time of digestion is raised from twenty-one to fifty-two hours; but at 25 dynes/cm. the increase is less than two-fold.

A better idea of the distribution of the pressure-soluble components can be had from Table IV, which gives the successive increments in the permanent changes of area expressed as percentages of the initial area of the undigested protein. For example, according to Table III, the film from the solution which had been digested for twenty-one hours gave a 44% decrease in area at 20 dynes/cm., while at 15 dynes/cm. the decrease was 30%. The increment in the amount of the pressure-soluble substances which were forced into solution by raising the pressure from 15 to 20 dynes/cm. was thus 14% of the initial area of this film, which in turn was only 54% (*i. e.*, 0.31/0.57) of the area of the undigested protein. Thus the increment of the actual amount of the pressure-soluble substance represents 7.6% of the film obtained from the undigested protein. This figure is given in the third column of Table IV.

The first line of Table IV (for $F = 1$ dyne/cm.) gives the amounts of soluble substances produced by digestion; *i. e.*, substances which are driven into solution by the application of a pressure of 1 dyne/cm. The last line of the table which is marked "Residue" shows the total amounts of protein remaining in the film after exposure to 25 dynes/cm. These values represent the pressure-insoluble components.

TABLE IV

A COMPARISON OF THE AMOUNTS OF SOLUBLE AND PRESSURE-SOLUBLE COMPONENTS OF PARTLY DIGESTED INSULIN THAT ARE DRIVEN INTO SOLUTION BY EXPOSURE TO INCREASING PRESSURES

F, dynes/cm.	Time of digestion in hours 0	21	52
1	0%	46%	70%
5	0.7	1.4	4.5
10	0.4	4.0	7.7
15	3.7	10.9	8.6
20	5.2	7.6	4.5
25	3.0	4.3	3.0
Residue	87.0	25.8	1.7

By plotting the data in the three columns of Table IV in terms of F as abscissa, it is seen that the maximum in the zero-hour curve occurs at $F = 19$, in the twenty-one-hour curve at $F = 16$, and in the fifty-two-hour curve at $F = 13$. The pressure-soluble substances produced by digestion thus become gradually more easily pressure soluble as the digestion proceeds. This suggests that digestion products are themselves digested so that a progressive decrease in molecular weight occurs.

Tiselius and Eriksson-Quensel[20] in recent studies of the digestion of egg albumin by pepsin have concluded that the products of the digestion contain only unchanged large molecules and fully digested end-products of molecular weight 1080. Our results show that the digestion of insulin gives a series of products of progressively decreasing molecular weight.

The compressibility curve obtained with the well-aged "residue" from the twenty-one hours of digestion of insulin agreed closely with the compressibility curve of the undigested insulin. This suggests, although it does not prove, that the residue consists of unaltered insulin monolayer.

Heat Denaturation of Insulin.—A 2% solution of insulin hydrochloride in distilled water, heated at 100° in a water-bath for thirty minutes, forms a birefringent thixotropic gel having a *p*H of 2.7.

(20) A. Tiselius and I. B. Eriksson-Quensel, *Biochem. J.*, **33**, 1752 (1939).

This, when cooled to room temperature, still flows for a time after shaking. A portion, diluted to 0.33% and spread, produces a film which gives the same specific area at 1 dyne/cm., the same compression loss after 25 dynes/cm., and the same compressibility curve as are obtained for unheated insulin. This indicates that heating has not broken the insulin molecule into lower molecular weight products similar to those obtained by pepsin digestion. The formation of the stable birefringent gel shows, however, that the insulin has been modified to give anisodiametric micelles. Thus, after dilution of the 2% gel, to give a 0.7% protein solution, the static birefringence disappears but is replaced by strong birefringence of flow. A detailed investigation of the production of birefringence in insulin solutions is being planned.

After four days of digestion with pepsin (0.01 mg./ml. at 38°) this gel shows no loss of birefringence or thixotropic behavior. Four hours of digestion produced no change in specific area at 1 dyne/cm., or compression loss at 25 dynes/cm., although other experiments with unheated insulin solutions showed marked decreases in area within this time. Our results therefore indicate that while unheated insulin solution in distilled water is easily broken down by pepsin a similar solution after heating to 100° is no longer digested. Glass electrode measurements show that 2% solutions of insulin hydrochloride in distilled water, heated or unheated, have almost the same *p*H of about 2.6.

The stiff thixotropic gel formed by heating a 4% insulin hydrochloride solution was diluted with an equal volume of 0.1 *N* hydrochloric acid, *p*H 1. Half of this was seeded with pepsin, the other half was used as a control. After forty-eight hours at 38° the seeded tube showed only a trace of birefringence while the control showed the original strong birefringence. Thus at *p*H 1, but not at *p*H 2.6, the thixotropic insulin gel can be digested with pepsin.

In another experiment half of a 2% insulin solution in 0.012 *N* hydrochloric acid was seeded with pepsin, and this tube and the control tube were allowed to digest at 38° for twenty-eight hours. During this time no increase in viscosity in either tube was observed, although we had previously observed that digestion in distilled water solution (*p*H 2.6) gave an increasing viscosity which finally resulted in a thixotropic gel. After twenty-eight hours both tubes were heated at 100° for twenty minutes. The viscosity of the seeded solution remained low during and after the heating and no birefringence could be observed. In the control tube, in which pepsin was omitted, heating produced a birefringent thixotropic gel. Evidently, therefore, the insulin was digested by the pepsin so that it was no longer able to form a thixotropic gel on heating.

Digestion of Egg Albumin.—A 0.33% solution of egg albumin in distilled water (*p*H 5.8) was almost unaffected by pepsin. Samples of 0.33% solution in hydrochloric acid at *p*H 2.0 gave the data in Table V.

TABLE V

DIGESTION OF EGG ALBUMIN WITH PEPSIN AT *p*H 2.0 AND 38°

Digestion time in hours	Specific areas at 1 dyne/cm.	Compression loss after 10 min. at 25 dynes/cm., %
0.0	0.63	4
.66	.543	3
2.0	.48	10
4.2	.34	15
6.5	.23	20
21.5	.036	..

Comparison with Table III shows that the composition of the films which spread from the partly digested egg albumin solutions contain only relatively small proportions of pressure-soluble components. Thus, with egg albumin 4.2 hours of digestion lowers the specific area at 1 dyne/cm. by 46% (0.63 to 0.34) and causes a compression loss of 15%, while with insulin a 46% reduction in specific area (after twenty-one hours of digestion) corresponds to a compression loss after 25 dynes/cm. of 52%. These results with egg albumin are thus in reasonable accord with the observations of Tiselius and Eriksson-Quensel on the digestion of this protein.[20]

Heat Denaturation of Pepsin.—The foregoing experiments on the pressure-soluble components of denatured proteins were made before we understood the relation between the (F, a) curves and the presence of pressure-displaceable substances. V. J. Schaefer has kindly made some measurements for us of (F, a, t) curves, like those shown in Fig. 6, using, however, monolayers from native and heat denatured pepsin. The results are summarized in Fig. 11. Because of the very large relative change in area, nearly six to one, it has been found desirable to use a logarithmic scale in plotting the abscissas, which represent the areas. This also has the advantage that the slopes of the

curves are independent of the amount of protein spread.

Curve A is a reproducible (F, a) loop given by a well-aged film from crystalline pepsin (Lehn and Fink). The monolayer was spread upon distilled water, pH 5.8, by the band method, using a 0.1% pepsin solution in water acidified with hydrochloric acid to give pH 2.

Another portion of the same 0.1% pepsin solution was denatured by heating for ten minutes to 63°. This solution gave an expansion pattern of the circular type with a strong edge effect which had been found to be characteristic of heat denatured pepsin.[4] The area of the expansion pattern at $F = 0$ produced from a given amount of denatured pepsin was considerably greater than that obtained from the same amount of native pepsin, but the relative areas were in the reverse order if the films were compressed to about $F = 1$ dyne/cm. Thus denaturation produces a relatively large amount of substances so pressure soluble that they go into solution at less than 1 dyne/cm., leaving only relatively little of the components that can withstand a higher pressure. Curve B in Fig. 11 is the initial (F, a) curve given by the standard (F, t) cycle, using two-minute time intervals. The permanent decrease in area resulting from this cycle amounts to 51%, as compared to 2% for crystalline pepsin (Table I), showing that the heat denaturation produced a very large amount of pressure-soluble substances.

A second (F, t) cycle with a maximum pressure of 23 dynes/cm. produced the (F, a) curve marked C, in Fig. 11. There is a further decrease in area, giving a total decrease of 72% of the original area. Subsequent cycles showed a continuing progressive decrease in area, so that with this denatured protein it did not seem possible to obtain closed (F, a) loops.

To study the effect of a more thorough pressure aging another film from the same solution of denatured protein was subjected to a pressure of 25 dynes/cm. for twelve minutes. During this time the fractional area decreased according to the equation $a/a_1 = 1.5/(t + 6.7)$, the final area being only 0.08 after $t = 12$ min. The results suggest that the film is not capable of standing pressure indefinitely. However, when the pressure was released, the area at 1 dyne/cm. increased within a few minutes to 0.32, perhaps because of diffusion of pressure-soluble substances back to the surface.

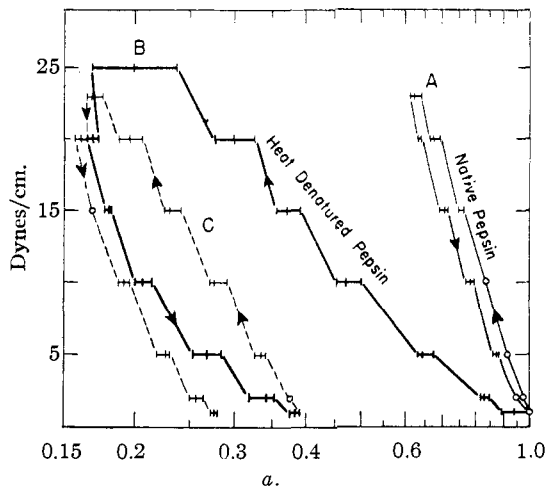

Fig. 11.—The effect of heat denaturation at 65° on the (F, a, t) curves given by monolayers from crystalline pepsin. The abscissas are plotted on a logarithmic scale.

The denaturation of pepsin by heat, which converts a large fraction of the protein into pressure-soluble substances, forms a striking contrast to the denaturation of insulin which upon heating gave no such products, although it did give them by digestion. The results suggest that the denaturation of the pepsin at 65° may have been due to self digestion.

These preliminary studies of (F, a, t) data given by films of denatured proteins demonstrate the general usefulness of this technique as a means of investigating protein denaturation.

Determination of Molecular Weights by Pressure-Solubility.—The data of Table II prove that pressure displaceability depends on the relative amounts of the various classes of amino acids. A measurement of this quantity thus serves to determine the average distribution of the residues along the polypeptide chain; the length of the chain should not be important. On the other hand, with a polypeptide chain of a given character, the pressure solubility must depend mainly upon the length of the chain.

A general theory of the effect of surface pressure in increasing the solubility of a monolayer was given in 1917.[21] This theory was based upon the Gibbs equation

$$dF/d \ln c = \sigma kT \qquad (7)$$

where σ represents the number of molecules/sq. cm. adsorbed at the air–water interface, and c is the concentration of the underlying solution which is in equilibrium with the film.

(21) I. Langmuir, THIS JOURNAL, **39**, 1883 (1917).

To calculate c as a function of F, it is evidently necessary to have one more equation involving the three variables, F, c, and σ. For this purpose, a hyperbolic adsorption isotherm based on an earlier empirical equation of Szyszkowski was chosen

$$c = A\sigma/(\sigma_1 - \sigma) \qquad (8)$$

where σ_1 is the value of σ for a close-packed film and A is a constant for a given substance. For very dilute solutions the ratio F/c approaches a limiting value which we may call $(F/c)_0$. This can be calculated from λ, the energy decrease involved in transferring a gram molecule of the substance from the solution to the interface. The relation can be written

$$\lambda = 1318 \log_{10} (F/c)_0 - 960 \text{ g. cal./mole} \qquad (9)$$

where c is expressed in moles/liter. For aliphatic substances of known composition it was shown that λ can be calculated by

$$\lambda = 625\,(n + 1) + \Sigma\lambda_0 \qquad (10)$$

where n is the total number of carbon atoms per molecule, and λ_0 represents the effect produced by each of the hydrophilic groups within the molecule. The -OH radical gives $\lambda_0 = -800$, while a -COOH radical gives $\lambda_0 = -938$.*

By combining Eqs. (7) and (8) we obtain

$$F = \sigma_1 kT \ln (1 + c/A) \qquad (11)$$

where A can be calculated by Eq. (8) or by

$$A = \sigma_1 kT/(F/c)_0 \qquad (12)$$

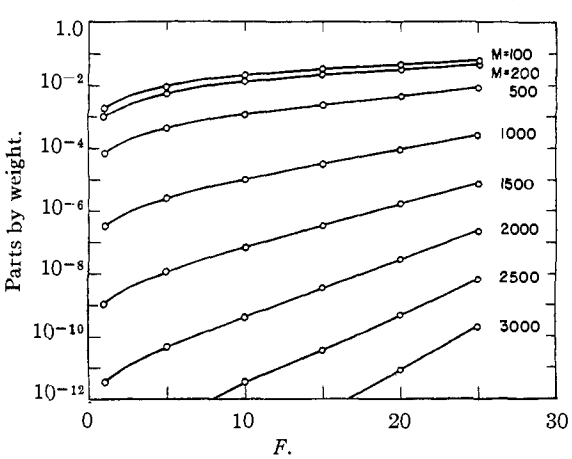

Fig. 12.—The pressure solubilities of typical polypeptides as functions of the surface pressure and the molecular weight, according to Eq. (25).

* The data for λ_0 given in 1917 are not applicable directly to long-chain polymers. If, however, we subtract 1375 from the values of λ_0 in Table IV of the 1917 paper and make the modifications which are incorporated in Eqs. (9) and (10) given above we calculate values for $(F/c)_0$ that are the same as those formerly obtained. But the equations are now in a form suitable for application to long-chain polymers.

To apply this theory to a long polypeptide chain, let us assume that the amino acid residues have an average molecular weight of 120. Thus, if the total molecular weight of a polypeptide chain is M, the number of residues is $M/120$. We may represent the repeating unit by —CO—CHR—NH—. If h is the effective number of carbon atoms in the side chains R, *i. e.*, the hydrophobicity of the side chains, we can place for the whole polypeptide chain

$$n = (2 + h)M/120 \qquad (13)$$

For the CO group $\lambda_0 = -1080$, and for NH $\lambda_0 = -690$. Substituting these values in Eq (10), we have

$$\lambda = 625 + (5.20\,h - 4.33)\,M \qquad (14)$$

From Schmidt's tables of analyses of proteins,[11] using our classification of the acids according to their hydrophobicity, we find that the values of h are: gliadin, 1.78; edestin, 2.01; zein, 2.30; egg albumin, 2.28; and insulin, 2.51; the average is 2.18. Inserting this in Eq. (14) and combining with Eq. (9) we obtain

$$\log_{10} (F/C)_0 = 1.202 + 0.00533\,M \qquad (15)$$

To determine A by Eq. (12) we now need to know $\sigma_1 kT$. We shall make the arbitrary and rough assumption that the films obtained by compressing polypeptide chains on a water surface have a specific area of 0.5 m.^{2}/mg. From this we calculate (at 20°)

$$\sigma_1 kT = 4800/M \qquad (16)$$

This can be substituted in Eq. (12) to get A and then F can be expressed as a function of c by Eq. (11). In dealing with protein solutions it is convenient to express concentrations in terms of w, parts by weight, instead of c, moles/liter. We therefore put

$$c = 1000\,w/M \qquad (17)$$

and Eq. (11) then becomes

$$\log_{10} [1 + 0.21w(F/c)_0] = FM/11{,}000 \qquad (18)$$

Figure 12 gives curves which represent this relation between w and f for a series of values of M.

The technique we have used for detecting pressure solubility is applicable only for a comparatively narrow range of values of w. For example, the protein in a solution having a concentration of less than 10^{-7} parts by weight diffuses to the surface so slowly that many hours are needed for the surface tension to reach an approximately constant value.[22] With solutions for which w =

(22) I. Langmuir and D. F. Waugh, *J. Gen. Physiol.*, **21**, 745 (1938).

10^{-5} the limiting value is reached relatively quickly. When the pressure solubility of a film consisting of breakdown products of proteins becomes greater than about 10^{-5}, all of the substance goes quickly into solution, even without the application of pressure. On the other hand, with pressure solubilities of 10^{-8} the total amount that could go into a solution 1 cm. deep, even under equilibrium conditions, could only be a small fraction of the material in the film. Experiments with other materials which we shall describe below have indicated that pressure solubility which is observable within a few minutes must be of the order of magnitude of $w = 10^{-6}$.

According to Fig. 12, polypeptide chains having the average composition of proteins should therefore show pressure solubility in the range of $F = 5$ to 10 when the molecular weight is about 1200, while if pressure solubility is observed between $F = 20$ and 25 the molecular weight is about 1700.

Many of the assumptions underlying the foregoing theory of pressure solubility are only rough approximations. For example, we have used the hyperbolic isotherm of Eq. (8). Recent theoretical studies of adsorption[23] have suggested that for molecules which act as the two-dimensional analog of elastic spheres the equation of state should be

$$F = \sigma_1 kT\Theta(1 + \Theta)/(1 - \Theta) \qquad (19)$$

where Θ is the covering fraction σ/σ_1. The corresponding adsorption isotherm is

$$\ln (c/A\Theta) = 2\Theta/(1 - \Theta) - 2 \ln (1 - \Theta) \qquad (20)$$

If we should use this isotherm in place of that of Eq. (8), Eq. (18) would have to be modified by adding another term, giving (for $F > 5$)

$$\log_{10} [1 + 0.21w(F/c)_0] = FM/11{,}000 + 2 \log_{10} (FM/9600) \qquad (21)$$

With values of w of the order of 10^{-6} the effect of this modification is to raise the calculated molecular weights by only 10 or 15%.

We have also neglected the fact that the pressure-soluble components compose only a part of the film. The true values of w, for given values of F and M, should thus be lower than those given by Eqs. (18) or (21). This effect should make the calculated values of M too large—a change in the opposite direction from that caused by the suggested adoption of the isotherm of Eq. (20).

(23) I. Langmuir, *J. Chem. Soc.*, 511 (1940).

A complete theory of pressure solubility would evidently be complicated and would require far more knowledge of the structure and properties of protein monolayers than we now possess. However, it is improbable that such a theory would change the values of w by a factor of more than one or two powers of ten, and thus by Fig. 12 should not alter the calculated molecular weights by more than 300 to 500 units.

The measurement of pressure solubility thus provides a method for the approximate determination of molecular weight, which becomes relatively more accurate for substances of high molecular weight, while most methods lose accuracy at high values of M. We may conclude that the pressure solubilities which we have observed with monolayers of native and denatured proteins are caused by products that have molecular weights in the range from 1000 to 2000.

To test this method of estimating molecular weights and to improve its accuracy we need to make measurements of the pressure solubilities of substances of known structure and molecular weight. We have made some preliminary experiments of this kind with monolayers of dioctyl sodium sulfosuccinate (Aerosol OT, 100%, made from 2-ethylhexanol by the American Cyanamid Company).

Films were spread by applying a benzene solution from a micropipet to the surface of water. When subjected to a pressure of 15 dynes/cm., the area of the compressed film on pure water decreased about 30% per minute. In other experiments the trough was filled with a solution of 7.7 mg. of Aerosol per liter ($c = 17 \times 10^{-6}$ M). The surface balance was placed near the middle of the tray, and the surface on both sides was then scraped clean. On one side of the surface balance the Aerosol which diffused to the surface was allowed to accumulate, while on the other side the surface was frequently scraped by a barrier so as to keep $F = 0$. In this way the gradually increasing pressure exerted by the accumulating film was measured. When the pressure had risen to about $F = 5$, the barrier was moved so as to produce and maintain any desired pressure F. To hold F constant required a progressive movement of the barrier. With $F = 5$ there was a steady increase in area of 4.2% per minute; at $F = 7$, + 2.2% per minute; at $F = 10$, a decrease of 1.9%; and at $F = 13$.

a decrease of 6.3% per minute. By interpolation the rate 0 corresponds to $F = 8.6$ dynes/cm.

Because of these changes with time it was not possible with this substance on pure water to obtain satisfactory (F, a) curves. However, with a substrate of 10^{-2} M barium chloride or saturated sodium sulfate the solubility became negligible, and the specific area extrapolated to $F = 0$ was 1.2 m.^{2}/mg., which corresponds to a value of $\sigma_1 kT = 4.5$. From these data, using in succession Eqs. (11), (12), and (9) it was found that $\lambda = 7400$. The composition of the Aerosol is $(C_8H_{17}COO)_2C_2H_3SO_3Na$. Allowing $\lambda_0 = -905$ for each ester group Eq. (10) gives $\lambda_0 = -4000$ for the sulfonate group. This value differs from that for the -COOH group by -3000, indicating that the increase in solubility caused by replacing -COOH by $-SO_3Na$ is the same as that given by shortening the hydrocarbon chain by 5 carbon atoms.

These experiments show that when the equilibrium value of w corresponding to a compressed film of Aerosol ($M = 444$) exceeds the actual concentration of the substrate by $\Delta w = 10^{-6}$, the rate of solution of the film is about 0.8% per minute. This result justifies our choice of 10^{-5} to 10^{-7} as the range in w in which pressure solubility is easily measurable by our technique involving time changes in the area of compressed monolayers.

We had hoped by detailed studies of the properties of Aerosol films to check the accuracy and applicability of Eqs. (9) to (12), and in this way if necessary to derive better equations. The experiments, however, showed that, contrary to reported properties of sulfonates and sulfates,[24] these monolayers of Aerosol are sensitive to even the minute amounts of divalent cations which are present in good grades of distilled water (10^{-7} M). A trace of a barium or lead salt purposely added to the water greatly increased the limiting value of F given by a monolayer formed upon a solution of Aerosol containing 5 mg. per liter.

The experimental difficulties introduced by this great sensitiveness are such that we consider this substance unsuitable for testing these equations. We are now conducting much more promising experiments using dipropyl and dibutyl sebacates, which are insensitive to impurities in the substrate and show easily measurable pressure solubilities.

(24) E. Stenhagen, *Trans. Faraday Soc.*, **36**, 496 (1940).

The method that we have described for estimating molecular weights from measurements of pressure solubility is applicable only to substances having molecular weights lying within a rather narrow range. Preliminary studies have shown, however, that this range can be greatly extended by altering the substrate upon which the film is spread. The addition of salts in fairly high concentrations produces a salting-out effect and causes a large increase in the values of λ and so makes the method applicable to substances of much lower molecular weight. For example, we find that di-isobutyl sodium sulfosuccinate (Aerosol IB) when spread on a saturated sodium sulfate solution gives films that have about the same pressure solubility as the films of the dioctyl compound spread on pure water.

The addition of salt may also be useful in studying pressure displaceability. The dashed curve in Fig. 9 shows the effect of introducing, under a film of wheat gliadin, enough ammonium sulfate to give a 20% solution. At high values of F the film area was unchanged, but at $F = 1$ dyne/cm. the area decreased 28%.

By the addition of water-soluble substances such as carbitol or butylene glycol that increase the solubility of hydrophobic groups, it is possible to decrease λ, and so permit the measurement of pressure solubility with substances of still higher molecular weights.

We wish to express our indebtedness to Mr. V. J. Schaefer for obtaining for us the data represented by Figs. 5, 6, 10, and 11.

Summary

The colored expansion patterns produced by spreading a small amount of indicator oil within a protein monolayer on water sometimes show an edge effect or discoloration near the outer boundary. This may be caused by unspread protein molecules in the substrate, but by passing the monolayer repeatedly over submerged barriers this unspread protein may be removed. When such a scrubbed monolayer is subjected to a pressure of 10 to 30 dynes/cm. for ten minutes or more, certain pressure-soluble components are forced into solution and may be detected and measured by the application of indicator oil to the water after scraping off the protein monolayer. This loss of material from the film is accompanied by, and is in quantitative agree-

ment with, a decrease in the area of the monolayer, measured under a standard compression of 1 dyne/cm.

After the monolayer has been well-aged by an application of pressure, $F = 25$ to 30 dynes/cm., of sufficient duration to drive out all pressure-soluble components, the force–area curves, (F, a), form reproducible closed loops, provided the pressure is raised and lowered according to a standard (F, t) cycle.

At $F > 15$ dynes/cm. sudden increases or decreases in F produce only slight changes in area, but these are followed by gradual changes of larger magnitude. These effects indicate that the apparent large compressibility of protein films results from a squeezing out of certain weakly hydrophobic amino acid residues from positions at the air–water interface into an underfilm where they contribute little to the surface pressure, although they are still attached through the polypeptide chains to the more strongly hydrophobic residues that remain in the overfilm.

This theory of pressure displaceability has led to a correlation of the compressibility curves of proteins to their chemical composition. The amino acids with side chains having a hydrophobicity less than that of $—C_2H_5$ determine the compressibility in the range from $F = 1$ to $F = 3$, while those more hydrophobic determine the areas at $F = 25$.

Films from denatured or partly digested proteins give large proportions of pressure-soluble components. This method of observing time changes in (F, a) curves provides a useful means of studying degradation products of proteins and obtaining information as to their molecular weights.

A general but preliminary theory of pressure solubility is given which is tested by studies of monolayers of Aerosols (dioctyl sodium sulfosuccinates). It indicated that the pressure-soluble components that were detected among the degradation products of proteins have molecular weights ranging from 1000 to 2000.

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[CONTRIBUTION FROM ALLERGEN INVESTIGATIONS, BUREAU OF AGRICULTURAL CHEMISTRY AND ENGINEERING, U. S. DEPARTMENT OF AGRICULTURE, AND THE ALLERGY CLINIC OF PROVIDENCE HOSPITAL, WASHINGTON, D. C.]

The Chemistry of Allergens. III. The Solubility Behavior of an Active Protein Picrate from Cottonseed[1]

BY JOSEPH R. SPIES, HARRY S. BERNTON AND HENRY STEVENS

Correlated chemical, clinical and immunological investigations[2] have shown that the principal allergenic component of cottonseed embryo is concentrated in the previously described protein picrate fraction CS-5.[2a] The unusual combination of chemical and physiological properties of the active component of CS-5 prompted an exhaustive study to determine whether the allergenic activity of this fraction is inherent or due to and unrecognized contaminant present in minor proportions.

An attempt to test the chromatographic homogeneity of CS-5 by solubility studies led to the development of a new method of fractionating the protein picrate and to the elucidation of some fundamental facts regarding the solubility behavior of a protein picrate. Although solubility measurements have found considerable application in determining homogeneity of proteins, the method has not been previously applied to a protein picrate.

To obtain solubility curves which would provide an evaluation of homogeneity, a weighed sample of CS-5 was equilibrated with successive volumes of solvent at constant temperature. The total nitrogen content of each extract was plotted against total per cent. of nitrogen removed from the original sample. It was assumed that a single component protein picrate, of the type represented by CS-5 or its fractionation products, would behave as a phase in equilibrium with saturated solutions according to equation (1)

$$\underset{\text{Solid}}{AP_x} \rightleftharpoons \underset{\text{Dissolved}}{AP_x} \rightleftharpoons AP^{+}_{(x-1)} + P^{-} + AP^{++}_{(x-2)} + P^{-} \quad \ldots (1)$$

(1) Presented in part at the 98th meeting of the American Chemical Society held at Boston, Massachusetts, September, 1939. Original manuscript received January 5, 1940. Not subject to copyright.

(2) (a) Paper II of this series: THIS JOURNAL, **62**, 1420 (1940). (b) Bernton, Spies and Stevens, "The Evidence of Multiplicity of Allergens and Reagins in Cottonseed Sensitiveness," *J. Allergy*, in press. (c) Coulson, Spies and Stevens, "The Immunochemistry of Allergens. I. Antigenic Properties of an Active Protein Component of Cottonseed," to be published.